Temporal Changes in the Muscle Extracellular Matrix Due to Volumetric Muscle Loss Injury Promote Functional Fibrosis

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Abstract

Skeletal muscle exhibits a remarkable ability to adapt and recover from a variety of stressors placed upon it. For example, numerous models of muscle injury, such as freeze, crush, and myotoxin, display immediate robust physical damage to the tissue when observed histologically. Yet in all three models the damaged tissue is able to completely regenerate back to pre-injury levels of fiber numbers and function. A complex process of cellular signaling mediates the successful regeneration seen in these models, which is ultimately achieved through satellite cell proliferation and fusion into new and existing myofibers. However, in more severe cases of traumatic injury, such as volumetric muscle loss (VML), there is a degree of damage that cannot be overcome endogenously. Instead of muscle regeneration, VML results in extensive fibrotic deposition and prolonged inflammatory signaling that dramatically reduces muscle function. The persistent fibrotic (TGF-β1, CCN2/CTGF) and inflammatory (IL-6, TNF-α) signaling further limits the ability of current treatment options to make significant improvements in patient outcomes. Moreover, the temporal changes in these processes have not been well documented following VML injury. Therefore, a study design using terminal time points of 3, 7, 14, 21 and 48 days following VML injury was created to evaluate the changes in extracellular proteins leading to fibrosis. Briefly, an initial significant increase in the proportion of collagen III was observed, which was subsequently reduced and overtaken by collagen I after 48 days post-VML. Similarly, histological results indicated an increase in loosely packed collagen through 21 days, before switching to an overwhelmingly dense collagen matrix by 48 days post-VML. Taken together, the window for successful intervention is likely before this shift to densely packed collagen I. Future work will evaluate immediate and delayed CCN2/CTGF inhibition combined with minced muscle graft implantation post-VML injury for effectiveness as interventions. Furthermore, the relationship between impaired neuromuscular junctions and fibrotic signaling will be assessed.

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Chapter 1: Review of the Literature

Skeletal Muscle Overview

Skeletal muscle is one of three muscle types in the human body, and makes up roughly 40% of total body weight. From a mechanical standpoint, the main function of skeletal muscle is force generation, which is transmitted through tendons to bones, ultimately causing movement of the appendicular skeleton. Skeletal muscle also aids in maintaining posture and breathing. From a metabolic standpoint, skeletal muscle acts as a reservoir of amino acids and carbohydrates, in addition to producing heat for thermoregulation. The other forms of muscle, cardiac and smooth, also generate force but are responsible for various involuntary processes such as contraction of the heart and digestion, respectively. Skeletal muscle exhibits a large degree of plasticity, or the ability to adapt both positively and negatively to different stressors or conditions. This is quite important, as skeletal muscle can easily sustain minor acute injuries or become overstressed due to the demands of everyday life but is expected to completely return to the prior state.

Introduction to Skeletal Muscle Growth and Development

The development of skeletal muscle, or myogenesis, begins before birth in the mesoderm of an embryo. Mesodermal cells take on a skeletal muscle lineage through a process called determination, whereby undifferentiated cells are programmed to various cell types in the presence of certain molecules and the right environment. Myogenic regulatory factors (MRFs) aided by PAX3 and PAX7 are critical for skeletal muscle development, by prompting the determination of undifferentiated mesodermal cells to myoblasts, or skeletal muscle precursor cells. Specifically, the MyoD family of transcription factors are thought to be the most important factors in the determination and eventual differentiation of myoblasts to myotubes, and are expressed exclusively in skeletal muscle. Expression of MyoD and Myf5 in somatic cells are an early indication of myogenic determination,[1] while it has been shown in the absence of these factors cells do not take on a myogenic fate and the organism cannot survive after birth.[2]

Following myoblast determination, differentiation is dependent on myogenin and MRF4,[2] in addition to another family of regulatory factors called myocyte enhancer factors (MEFs). The differentiation process involves proliferation of myoblasts that fuse together creating a multinucleated cell known as a myotube. Myotubes are non-mitotic; they grow and regenerate by the addition of protein and nuclei to the existing cell, rather than duplication. Maturation of myotubes to myofibers begins with the expression of cytoskeletal proteins, followed by myofibrillar proteins such as myosin heavy chain (MyHC). The maturation process is completed by the innervation of the fiber via the terminal axon of an α-motor neuron. This final step is critical, as without innervation the fiber will die.

Positive Regulation of Skeletal Muscle Growth

It is believed that the number of muscle fibers at birth is roughly the same number of fibers that a person will have throughout their lifespan. Muscle growth, or hypertrophy, is the result of adding contractile protein content to the cell, whereas muscle loss, or atrophy, results from the degradation of contractile proteins. The creation of entirely new skeletal muscle fibers after birth, known as hyperplasia, is thought to occur to a very limited extent in humans. Therefore, muscle growth results from increasing the size of existing muscle fibers, not the addition of new muscle fibers. Specifically, growth generally involves increase in muscle fiber diameter, or cross-sectional area (CSA), rather than length, although increase in fiber length can occur to some extent.

Skeletal muscle growth is achieved by a variety of anabolic processes regulated primarily by hormones and endogenous growth factors, which in part work to increase protein synthesis. Considered most upstream in this process, growth hormone-releasing hormone produced in the hypothalamus stimulates growth hormone (GH) secretion by the anterior pituitary gland. Growth hormone then increases production of insulin-like growth factor 1 (IGF-1), by binding to growth hormone receptors mainly in the liver, but also in skeletal muscle among other tissues. Hepatic production of IGF-1 spreads systemically, causing growth

stimulating effects throughout the body, however it is thought that local IGF-1 production is sufficient for skeletal muscle hypertrophy.[3]

Although the entire pathway is quite complex, it is accepted that IGF-1 binding to its receptor activates phosphatidylinositol-3-kinase (PI3K), leading to the phosphorylation of protein kinase B (Akt)[4]. Subsequently, Akt activation indirectly leads to perhaps the most important downstream factor of the pathway, known as the mammalian target of rapamycin (mTOR), a serine/threonine kinase. Specifically, mTOR is the common subunit of two larger complexes, termed mTORC1 and mTORC2, of which mTORC1 is implicated in processes of skeletal muscle growth. In a classic signaling cascade, the activation of mTORC1 leads to the phosphorylation and activation of S6K1,[5,6] in turn increasing protein synthesis by the phosphorylation and activation of the ribosomal protein S6 (rpS6). rpS6 is part of the small 40S ribosomal subunit and is thought to be involved in translation of mRNA to protein.

Testosterone is another well-known hormone with overall anabolic effects leading to skeletal muscle hypertrophy. However, the exact mechanism by which it facilitates these results has been somewhat unclear. Testosterone has been mainly thought to have genomic effects, increasing DNA transcription of pro-growth factors after binding to an androgen receptor.[7] Hence, these effects are not immediate, but rather take hours or days. But similarly to GH and IGF-1, it is now believed that mTOR becomes activated downstream of testosterone, producing rapid effects.[8] Unlike the IGF-1 pathway, it appears Akt is not necessary for testosterone mediated mTOR activation. Rather, testosterone can activate the MAPK/ERK pathway, which has genomic effects by activating transcription factors, but also indirectly activates mTOR.[8] A proposed pathway for this is phosphorylation of tuberous sclerosis complex 2 (TSC2), a protein that works to inhibit mTORC1, by ERK1/2. Phosphorylation by ERK1/2 is inhibitory of TSC2 and so it is believed to help activate mTORC1.[9] Interestingly, recent studies have shown that increases in the amount of circulating testosterone (within the normal physiological range) is not indicative of proportional increases in strength or muscle growth, but rather the amount of androgen receptors is.[10] Therefore, it

appears the sensitivity to testosterone is more important for muscle growth than the overall amount of the hormone produced.

A crucial yet complicated step in the process of protein synthesis involves the initiation of mRNA translation. There are over a dozen eukaryotic initiation factors, all modulating the process in some form.[11,12] However, in general the inhibition of an initiation factor will impede translation. Thus, another way mTORC1 increases protein synthesis is by the phosphorylation of 4E-binding protein 1 (4EBP1), causing it to dissociate from the translation initiation factor 4E (eIF-4E).[5,6] When 4EBP1 is bound to eIF-4E, translation of mRNA is repressed, and therefore dissociation of 4EBP1 can increase translation and protein synthesis. In a similar manner, insulin is important for protein synthesis by inhibition of glycogen synthase kinase 3 (GSK3) through activation of the PI3K/Akt pathway. Although GSK3 was named for its role in limiting glycogen synthesis, it is now known to have numerous effects. Phosphorylation of initiation factor 2B (eIF-2B) by GSK3 suppresses its activity,[9] however Akt prevents this by phosphorylating GSK3.

Insulin and IGF-1 also appear to suppress protein breakdown by inhibiting the ubiquitin ligases atrogin-1 and MuRF1 through the same PI3K/Akt pathway.[13] Akt phosphorylates FoxO, inactivating the transcription of atrogin-1 and MuRF1.[4] Simply put, these molecules work by labeling targeted proteins with ubiquitin for subsequent proteasomal degradation. Therefore, the effect of insulin and IGF-1 in skeletal muscle is two-fold, they stimulate the rate of protein synthesis while simultaneously slowing the rate of protein degradation, effectively increasing muscle growth. At all times throughout the lifespan there is protein turnover consisting of concurrent synthesis and degradation. The net balance of these two processes plays a large role in determining whether skeletal muscle is achieving hypertrophy, atrophy, or maintaining size. Such that a higher rate of protein synthesis compared to degradation results in positive protein accretion and muscle growth.

Negative Regulation of Skeletal Muscle Growth

Skeletal muscle growth is negatively regulated by members of the transforming growth factor beta (TGF-β) family, namely myostatin. The mechanisms by which myostatin exerts its negative influence on muscle growth are thought to be numerous. In relation to protein synthesis pathways, myostatin may inhibit Akt,[4,14] which concurrently limits mTORC1 activation and increases FoxO activation. In opposition to insulin and IGF-1, this would decrease protein synthesis and increase degradation by increasing atrogin-1 and MuRF1 expression. Myostatin most likely exerts these effects by phosphorylation of Smad2 and 3 transcription factors through binding to activin type IIB receptors.[4,15,16]

Research on this topic has been helped in part by rare yet naturally occurring deletions of the myostatin gene in numerous mammals, including humans.[17,18,19] In all cases, skeletal muscle mass was significantly increased compared to typically developing members of the given species. Notably, McPherron et al. showed that in myostatin knockout models of mice, the increased muscle mass was due to a combination of hyperplasia and hypertrophy.[20] This has led to the belief that myostatin regulates skeletal muscle growth by two separate mechanisms. Indeed, manipulation of myostatin expression in different transgenic models leads to differential muscle growth in terms of hypertrophy and hyperplasia.[21,22] In agreement with Lee,[16] it would appear myostatin has distinct roles in regulating prenatal muscle fiber myogenesis and postnatal muscle fiber growth. The resulting increase in hyperplasia or hypertrophy would be entirely dependent on the timing of myostatin inhibition. Reduced myostatin expression during embryotic development would result in increased fiber number, but not necessarily size; while reduced myostatin expression after birth would result in increased fiber size, but not necessarily increased fiber numbers.

Recent work has also implicated the increase in muscle mass from myostatin knockout models is accompanied by a decrease in the relative proportion of extracellular matrix (ECM) proteins surrounding muscle fibers.[23] Likewise, Li et al. found that myostatin increases proliferation of muscle fibroblasts *in vitro* and *vivo*.[24] The dual role of myostatin as an inhibitor of muscle growth and stimulator of ECM

growth makes it an intriguing therapeutic target. However, it appears the regulation of muscle growth by myostatin is highly conserved and serves an overall beneficial purpose.[16] Knockout models of myostatin in mice have shown the typical increase in muscle mass, yet somewhat differ in results regarding maximal force production.[25,26] Amthor et al. found no increase in overall maximal force between wild-type and myostatin knockout mice,[25] while Mendias et al. found an increase in maximal force following myostatin knockout.[26] Yet, when adjusted for the size of the muscle (i.e., specific force), both studies found a reduced force output in the myostatin knockout groups, suggesting myostatin plays a role in maintaining the efficiency of force production. This is potentially due to the weakening of tendons with myostatin inhibition, as suggested by Mendias et al.[27] Compared to wild-type controls, tendons from myostatin knockout mice obtained a higher peak stress, but less than half of the peak strain before yielding, indicating a more brittle tendon. Interestingly, they also exhibited a higher stiffness compared to wild-type, even though overall collagen content was reduced.[27] Thus, the probability of injury is likely increased with larger muscle mass and weaker tendons. In addition, oxidative capacity is reduced in genetic loss of myostatin, most likely due to a reduction in overall mitochondria content.[25] Therefore, myostatin is most likely a healthy regulator of muscle mass in the typical population, but its inhibition could still be a useful target in certain populations characterized by muscle atrophy and increased fibrosis.

External Factors Regulating Skeletal Muscle Growth

External factors contributing to muscle growth include nutrition, exercise, and the overall whole body energy state. A fasted or starved state (i.e., low-energy) promotes overall catabolism, as the body needs to break down macronutrients for energy to maintain homeostasis. Conversely, a fed state (i.e., high-energy) promotes the storage or build-up of macronutrients and therefore has an overall anabolic effect.[28] The influence of energy states on muscle growth is largely due to AMP-activated kinase (AMPK).[29] When energy levels are low, there is an increase in the amount of ADP and AMP relative to ATP, causing activation of

AMPK.[30] The end result of AMPK activation in this case is the increased production of ATP. But not only will activation of AMPK increase ATP production; it will simultaneously limit ATP usage for anabolic processes such as the synthesis of glycogen and protein, and thus muscle growth.[30] Specifically, AMPK inhibits protein synthesis by the direct phosphorylation of the mTORC1 subunit, Raptor.[31] Although oversimplified here, the ability of AMPK to sense the body's energy state can be thought of like a building's thermostat. When a building becomes too cold, the thermostat senses the drop in temperature and signals the furnace or heating system to produce warm air; just as when the level of ATP drops in the body, AMPK senses the rise in AMP and signals the breakdown of glycogen, lipids, and potentially proteins to produce more ATP.

Fatty acid oxidation, or β-oxidation, is the preferred energy system in skeletal muscle at rest, along with aerobic glycolysis. Protein is also capable of contributing to energy production; however, this is not ideal as it can result in muscle atrophy if utilized in a prolonged state. Additionally, when proteins are broken down to amino acids for fuel use, the amino group needs to be removed. In what is known as the glucose-alanine cycle, branched chain amino acids from muscle protein are converted to glutamate and ketoacids. Alanine transaminase then converts glutamate and pyruvate to alanine, which is shuttled to the liver (along with ketoacids) to produce glucose via the gluconeogenic pathway. The glucose made in the liver can then be shuttled back to the muscle for ATP production.[32] Importantly, once alanine or other amino acids reach the liver, the amino group can be removed in the form of urea and excreted. The use of protein as a fuel source is typically only necessary in times of stress, such as during intense exercise or an extended fasting period.

Exercise is a well-known modulator of muscle growth and metabolism, however it is an intricate process. Differing intensities and durations of exercise will play a large role on the outcome of overall anabolism or catabolism. Resistance exercise, or weight training, is a well-known stimulator of skeletal muscle hypertrophy and strength. Although an acute bout of high intensity weight training will increase muscle protein breakdown, it also increases protein synthesis post-exercise for up

to 48 hours.[33,34] However, without food intake before or after a training session, the overall protein balance will most likely remain negative,[33,34] highlighting the importance of nutrition to stimulate positive protein accretion and muscle hypertrophy. Interestingly, numerous studies have found that exercise regimens containing resistance training concurrently with endurance training exhibit dampened hypertrophy and strength gains compared to resistance training alone.[35,36,37] This could be because lower intensity, long duration exercise mimics the low-energy status produced by fasting, as ATP stores are gradually used up to produce continued muscle contraction and movement. Large volumes of endurance exercise generally result in a net protein loss if not accompanied by adequate nutrition,[38] due to an increase in protein breakdown for fuel use.[39] Although the degree of activation is dependent on exercise intensity, AMPK is thought to mediate this process.[40,41] Therefore, endurance exercise in a fasted state can be considered overall catabolic, hindering muscle hypertrophy and strength. However, it is difficult to make broad claims on this subject because of the numerous variables alluded to previously, including intensity, duration, frequency, training status and nutrition. Minor adjustments in any of these variables can produce differing results, as seen by the amount of conflicting studies on the subject.[36,42,43,44] Recently, Kazior et al. found that endurance exercise actually augments the effects of resistance training on skeletal muscle hypertrophy and mTOR activation.[43] Clearly, endurance exercise can elicit increases in skeletal muscle force output and hypertrophy to an extent, but is most likely dependent on concurrent increases in calories consumed, as is resistance training.

It is widely agreed upon that endurance training results in an increased oxidative capacity of skeletal muscle. The major mechanism responsible for this adaptation is the increase in mitochondrial content,[45] allowing greater fat oxidation and a more sustained utilization of glycogen.[46] Mitochondrial biogenesis is heavily regulated by the transcriptional factor peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). The upregulation of PGC-1α is achieved through a variety of pathways. The promoter region of PGC-1α contains binding sites for cAMP response element binding protein (CREB) and MEF2.[47] Exercise

induced increases in Ca^{2+} causes activation of calmodulin kinase (CaMK), which phosphorylates CREB, promoting transcription of PGC-1α.[48] Likewise, AMPK activation due to endurance exercise can increase CREB phosphorylation. Concurrent increases in hydrogen peroxide (H_2O_2) from endurance exercise activate p38 MAPK, which can phosphorylate MEF2 and thus also increase PGC-1α transcription.[49] Once translated, PGC-1α increases the replication of mitochondrial DNA through activation of mitochondrial transcription factor A (TFAM).

Altogether, skeletal muscle has a remarkable ability to adapt to different stressors placed upon it. In fact, such stressors are necessary to maintain and/or improve overall health of the muscle tissue. Certain molecular products that are typically considered harmful to tissue, such as H_2O_2, can elicit beneficial changes to skeletal muscle in a hormetic effect. Without these signals, muscle protein will be broken down for other uses and mitochondrial turnover will become stagnant, leading to muscle atrophy and reduced function.

Skeletal Muscle Repair and Regeneration

The repair and regeneration of existing muscle fibers follows a similar process as that of newly developing fibers outlined previously. Because myofiber nuclei are post-mitotic and therefore unable to re-enter a proliferative state, the activation of skeletal muscle stem cells, known as satellite cells, is crucial to the regeneration process. Satellite cells remain in a quiescent state beneath the basal lamina until activated upon stress and damage to the muscle.[50] An inflammatory response is triggered in response to this stress, which involves the influx of phagocytes and cytokines (IL-6, TNF).[51] These both work to clear necrotic debris from the site of injury. Activated satellite cells then proliferate at the inner basal lamina and fuse into multinucleated myotubes. These myotubes finally fuse with the existing myofibers and become innervated to complete the regeneration process, similar to that of newly developing muscle fibers.

An important biomarker of quiescent satellite cells in adult skeletal muscle tissue is the transcription factor paired box protein 7 (Pax7).[52] Activated satellite

cells increase in size and maintain Pax7 expression along with myogenic regulatory factors, which can include Myf5 and/or MyoD. Determination of activated satellite cells into myoblasts are then characterized by a loss of Pax7 expression and an increase in MRF expression including Myf5, MRF4, and MyoD. Finally, differentiation of myoblasts into myotubes and myofibers is highlighted by the presence of myogenin and MyHC in the continued presence of MRFs. Importantly, quiescent satellite cells ($Pax7^+/Myf5^-$) are able to self-renew, thereby repopulating the stem cell niche for continued regeneration.

There have been debates regarding the necessity of $Pax7^+$ satellite cells for skeletal muscle regeneration, as it appears other progenitor cells are capable of contributing to the process. Ferrari et al. first demonstrated that bone marrow-derived progenitors are able to migrate into skeletal muscle and assist in regeneration.[53] This was followed by studies showing numerous other cells can take on a myogenic lineage, including mesoangioblasts and pericytes.[54,55] However, in 2011 Lepper et al. determined that although other progenitors can assist with skeletal muscle regeneration, $Pax7^+$ satellite cells are a necessity, as complete elimination of them inhibits regeneration.[56]

Skeletal Muscle Contraction

Skeletal muscle contraction can be broken down broadly into two main phases, excitation-contraction (E-C) coupling and cross-bridge cycling. The process of E-C coupling begins with an action potential, or nerve impulse, sent from an α-motor neuron in the central nervous system to the muscle via descending axons. The impulse is propagated down the axon by a change in polarity caused by gated ion channels allowing Na^+ into the axon and K^+ out of the axon, until it reaches the neuromuscular junction (NMJ). The speed at which this impulse travels to the muscle is increased by the presence of myelin sheaths, which act as insulators to the nerve fiber. One motor neuron is responsible for innervating multiple muscle fibers, known as a motor unit. Smaller motor neurons are typically associated with smaller muscle fibers, known as slow-twitch, which are relatively fatigue resistant. Conversely, large motor neurons are associated with larger muscle fibers, or fast-

twitch, which produce more force but fatigue more quickly. According to the all-or-none principle,[57] when an action potential is initiated by a motor neuron, all of the fibers within its motor unit will contract together at its greatest strength, or if the stimulus is too weak it will not contract at all. There is no ability for a muscle contraction to occur at a degree less than maximal for that given motor unit. However, according to the size principle,[58] smaller motor units will always be the first to activate during a given task, and if more force is needed, larger motor units will be recruited in an orderly fashion. Because of this, one is able to control the amount of force output needed for a given task and not waste energy using large motor units for tasks that only require a small amount of force.

Once an action potential reaches the NMJ, it releases a neurotransmitter, acetylcholine, which binds to receptors on the sarcolemma.[59] The binding then activates an electrochemical gradient across the muscle membrane, similar to how the action potential was originally sent from the central nervous system. This can be thought of like a rock being tossed into a pond. The rock hits the water and the water ripples out in all directions from that point. However, the further from the point of contact, the weaker the ripples become. In terms of electrophysiology, this phenomenon is known as the length constant, which describes how the potential diminishes from a given point of event. The NMJ is akin to this point, whereby the signal begins and can spread across the muscle. But, unlike the ripples of water which diminish as they spread, ion channels located along the transverse tubule (T-tubule) network allow the potential to be propagated and maintain equal strength throughout the entirety of the muscle. This is extremely important as it allows muscles to produce a much larger force than they could without T-tubules maintaining the action potential.[60]

Located along the T-tubules are voltage-gated channels known as dihydropyridine receptors (DHPR), which are physically coupled to ryanodine receptors (RyR) on the sarcoplasmic reticulum (SR).[61] Depolarization of the T-tubule membrane caused by an action potential triggers the DHPR to activate the RyR, which allows calcium (Ca^{2+}) to be released from the SR. This process is the

final step in E-C coupling, as the release of Ca^{2+} ultimately triggers a muscle contraction.

As previously noted, the “second phase” in skeletal muscle contraction is known as cross-bridge cycling, and/or the sliding filament theory. Structurally, individual muscle fibers are divided into sections called sarcomeres.[62] Sarcomeres contain the two main contractile proteins contributing to the sliding filament theory, actin and myosin. On a simplistic level, muscle contraction is achieved by myosin pulling on actin, which shortens the length of each sarcomere and thus the muscle as a whole. The actin filament is anchored to the Z-disk and has some degree of overlap with myosin on both ends (**Figure 1**). As the myosin heads bind to actin

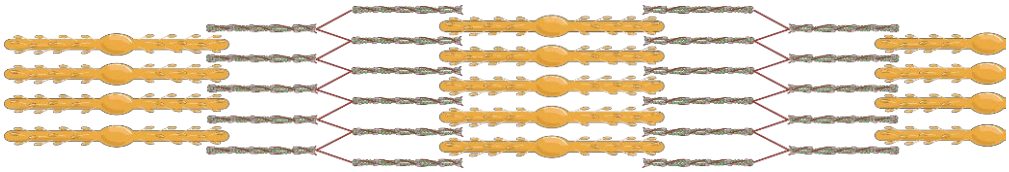

Figure 1. Structure of a sarcomere. Myosin (thick filament), actin (thin filament), Z-disk (anchoring thin filaments).
Figure was produced using Servier Medical Art (https://smart.servier.com)

on both ends, it pulls actin towards each other and causes the sarcomere, the length between two Z-disks, to shorten. A cross-bridge refers to a single myosin head bound to globular actin. During a muscle contraction, there is a constant cycle of myosin binding, pulling, and releasing from actin, hence the term cross-bridge cycling. A good analogy here is that of a rowing team, with the oars corresponding to myosin and the water actin. However, in skeletal muscle the individual myosin heads do not cycle in unison, but rather in an asynchronous fashion so that at any given time during a contraction there are both bound and unbound cross-bridges. This allows for fluid motion on a macro level. Perhaps rowing crews could be more efficient by alternating their strokes, in which case there would be a reduction in the amount of velocity fluctuation between each row.

Cross-bridge cycling is much more complicated upon closer examination. Myosin heads can only bind to actin at certain locations, which happen to be blocked during rest by the protein tropomyosin. In order for a cross-bridge to form, this binding site needs to be exposed to myosin. A protein called troponin, attached to tropomyosin, is responsible for making this happen. Troponin is a complex made

up of three proteins, troponin-T (TnT), troponin-C (TnC), and troponin-I (TnI). As mentioned previously, the final step in E-C coupling involves the release of Ca^{2+} into the sarcoplasm. Ca^{2+} then binds to TnC, causing a conformational change in the troponin-tropomyosin complex and exposing the actin binding site to myosin. Once the myosin head is bound to actin, it can then undergo a powerstroke, effectively shortening the sarcomere before detaching and repeating the process.

The biochemical side of the cross-bridge cycle is controlled by ATP, as it is needed for the release of myosin from actin after the powerstroke. Without ATP, muscles would remain in a rigor state which is seen acutely after death, as the body stops producing ATP and the myosin heads become stuck in a post-powerstroke state bound to actin. In healthy muscle however, ATP binds to myosin, causing it to dissociate from actin. Myosin then hydrolyzes the ATP, making it an ATPase itself, something not discovered until 1939 by Engelhardt and Ljubimowa.[63] The hydrolysis re-primes myosin to a pre-powerstroke state, ready to bind to actin once again, assuming Ca^{2+} is still present to expose the binding site. The products of hydrolysis, ADP and P_i, are released while myosin is bound to actin. First, the release of P_i from myosin results in the powerstroke and a more strongly bound state. ADP is then released post-powerstroke to allow for ATP to rebind and release the cross bridge. The cycle continues until Ca^{2+} is pumped back into the SR, which allows the muscle to relax. Thus, diminished Ca^{2+} release from the SR during intense exercise is generally thought to be a main contributor to muscle fatigue and decreased force output.

Length/Tension Relationship

As previously noted in **Figure 1**, actin is anchored to the Z-disk and overlaps with myosin. It is well understood that the amount of overlap is crucial to the potential force of muscle contraction, which is generally referred to as sarcomere length. The longer the sarcomere, the less overlap between actin and myosin, and therefore less myosin heads can be in contact with actin at the initiation of muscle

contraction. On the other hand, too much overlap means more myosin heads can be in contact with actin, however there is less space to contract before actin filaments interfere with each other. The optimal sarcomere length is somewhere in the middle, allowing maximal cross-bridges to be formed and enough room for forceful contraction without interference. The relationship between sarcomere length and tension can be shown by isometrically contracting muscle at various lengths. An isometric contraction is one that does not result in a change in length, but rather produces tension in a static position, often opposing some type of force, such as gravity. This differs from concentric contraction, which produces tension while shortening the muscle, and eccentric contraction, which produces tension while the muscle is lengthening. Examples of these include the biceps brachii during a dumbbell curl, and the quadriceps muscle group during the landing phase of a jump, respectively. The greatest tension an actively contracting muscle can achieve is when that muscle is at optimal length **Figure 2**. If the muscle is shortened or lengthened before actively contracting, the tension produced is drastically reduced. However, if the muscle is stretched past its optimal length, there is a strong resistance to that stretch which will produce tension, known as passive tension. Whereas active tension is due to the cycling of cross bridges and controlled biochemically, passive tension is caused by resistance of opposing force mainly due to the large protein titin. Titin is anchored to the Z-disk on one end, and the M-line, the center of the sarcomere, on the other end. Titin physically produces tension by having elastic properties, similar to a rubber band. At rest the protein has folded domains which unfold as the muscle stretches, providing tension. By equating titin to a spring under Hooke's Law, the amount it stretches is directly proportional to the force with which it pulls. Because of this, the total amount of

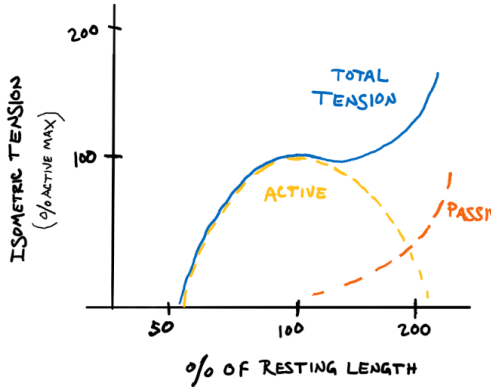

Figure 2. Hypothetical graph of length/tension relationship. Total tension (solid line) combines active and passive (dashed lines) increments when muscle is stretched beyond resting length.

tension a muscle can produce is increased by combining active and passive tension (solid line in **Figure 2**). As an easy example, one can lower a much heavier amount of weight to the ground than can be picked up off the ground. This is because while lowering weight controllably, the primary muscles are lengthening, and therefore force is being produced both actively and passively. When picking weight up off the ground, only active tension is produced, i.e., titin does not contribute to the force production.

Force/Velocity Relationship

Another important concept of skeletal muscle function to understand is the force-velocity curve, which has a somewhat inversely proportional relationship. That is, as the velocity of a concentrically contracting muscle increases, the force of contraction decreases, and vice-versa. If one were to map power on the same graph, which for a constant force and velocity equals force times velocity, it should have a relatively parabolic shape. Optimal power is achieved around roughly one-third of peak velocity, and moves towards zero as one approaches either maximal force or maximal velocity. In reality, the force-velocity relationship slightly differs for everybody and can be modulated by training, as shown in **Figure 3**. For example, the shape of the curve for an Olympic weightlifter versus an Olympic sprinter will likely be different. The weightlifter should exhibit more power near maximal force, while the sprinter should achieve greater power near maximal velocity.

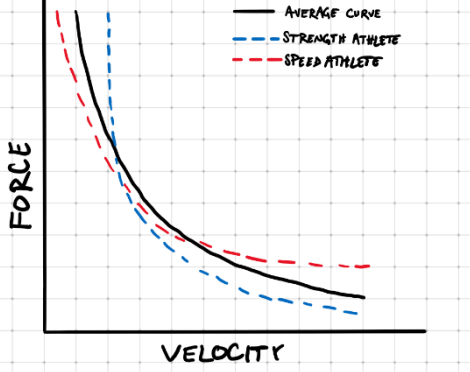

Figure 3. Hypothetical graph of force/velocity relationship.

Non-Contractile Aspects of Skeletal Muscle

In addition to the contractile proteins comprising skeletal muscle, there are also very important non-contractile proteins within and surrounding muscle fibers. Muscle tissue can generally be divided into layers based on connective tissue,

although rather subjectively. Single muscle fibers are surrounded by endomysium, bundles of muscle fibers (fascicles) are surrounded by perimysium, and the whole muscle unit is surrounded by epimysium (**Figure 4**).[64]

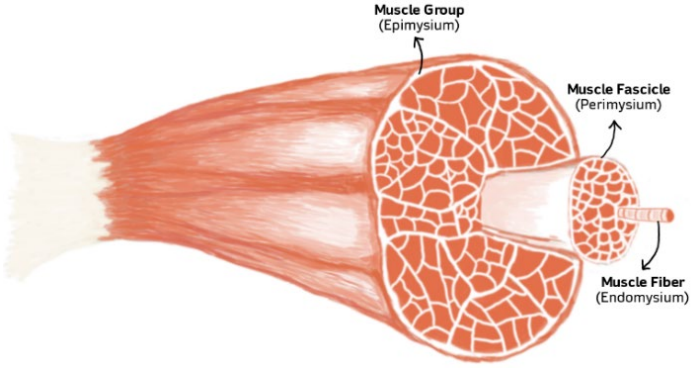

Figure 4. Skeletal muscle organization.

These layers of connective tissue are known as the extracellular matrix, or ECM. The ECM is predominately comprised of varying collagen isoforms, which physically provide a level of elasticity to the muscle and allows force transmission from muscle to bone to produce movement. Perhaps even more so than titin (described above), collagen is heavily implicated in the amount of passive tension produced by muscle fibers. There are currently 28 known types or isoforms of collagen, which is the most abundant protein found in all mammals.[65] Type I collagen is the most abundant isoform in skeletal muscle ECM. The perimysium layer and tendons are almost entirely collagen I, while the endomysium and epimysium layers are typically equal parts collagen I and III.[66] Type IV collagen, along with some type VI, makes up the muscle basement membrane. In this way, type IV collagen connects skeletal muscle fibers to the ECM, as the glycoprotein laminin binds to the collagen IV network and is also anchored to the dystrophin-glycoprotein complex spanning the sarcolemma. Dystrophin, along with many other structural proteins such as desmin, make up costameres physically coupling the sarcomere to the sarcolemma. Thus, the path of force transmission generated by actin and myosin moves through costameric proteins to the muscle membrane, to laminin and collagen IV, to endomysium and perimysium collagens I and III, to tendons, and finally bones. The entire process is not fully understood but generally accepted that force is transmitted close to this fashion. It is easy to see then, that the ECM plays quite an important role in optimal functioning of skeletal muscle.

Collagen is unique from other proteins in that it is made up of roughly 12.5% hydroxyproline. Because of this relatively high content compared to other proteins, hydroxyproline is commonly used as a biochemical marker for total collagen content. A single collagen molecule has a triple-helix structure made up of three polypeptide chains and is also referred to as a tropocollagen. Tropocollagens link together to form a collagen fibril, and collagen fibrils link together to form a collagen fiber, which can also link to form cables. These cross-links are formed by covalent binding of pyridinoline to lysyl and hydroxylysyl residues on the collagen molecules, catalyzed by the enzyme lysyl oxidase (Lox). Covalent cross-linking within varying levels of collagen fibrils and fibers is thought to contribute to the amount of stiffness observed in a given tissue. More cross-linking presumably leads to a more rigid fiber. It is generally believed that cross-linking increases as collagen matures, and therefore newly synthesized collagen will have less cross-linking. Additionally, cross-linking may play a role in the solubility of collagen, as fibers with more cross-links are harder or even impossible for collagenases, such as matrix metalloproteinases (MMPs), to degrade. However, much of this is still speculation as studies have shown mixed results.

Many aspects of the ECM are not as well understood as the muscle fibers they surround. Historically, skeletal muscle ECM has been thought of as a static environment giving support to muscle fibers, however recently it is beginning to be understood as an extremely important dynamic aspect of healthy muscle. For example, proteoglycans within ECM provide storage for growth factors and thus play a large role in cell signaling and remodeling. As the name suggests, proteoglycans consist of a core protein bound to a glycosaminoglycan (GAG) chain. The core protein typically binds to collagen at specific locations on the fibril. Considerable research has been done on the role of decorin and biglycan, which belong to the small leucine-rich repeat (SLRP) group of proteoglycans. Because of their hydrophilic nature, the most well-known function of these molecules is to provide hydration to the ECM, supporting the elastic properties of collagen. However, along with this general physical support by binding to collagen, decorin is thought to regulate collagen fibrillogenesis, preventing abnormal collagen fiber

formation. Genetic knock-out models of decorin and biglycan have resulted in increased collagen fiber diameter, as well as reduced circularity of collagen fibers in tendons.[67,68] Adding to its importance for a healthy ECM, decorin has been shown to bind with TGF-β family members, reducing the effects of myostatin and TGF-β1,[69,70] both pro-fibrotic proteins.

ECM Turnover – Synthesis & Degradation

The synthesis of ECM components is produced chiefly by fibroblasts. Fibroblasts reside within the ECM, which undergoes a continual remodeling from deposition of components by fibroblasts and degradation by enzymes such as MMPs. In a healthy regeneration model, damaged muscle fibers activate an immune response that recruits mast cells and various white blood cells to the area, such as neutrophils, monocytes, lymphocytes and eosinophils. Mast cells release histamine along with numerous cytokines, ultimately leading to the transition of monocytes to M1 macrophages. M1 macrophages are generally considered pro-inflammatory, secreting nitric oxide, tumor necrosis factor (TNF), and various interleukins, in addition to promoting proliferation of satellite cells.[71] Although these factors are important in the immediate response to a stressor, M1 macrophages will quickly transition to anti-inflammatory M2 macrophages. M2 macrophages play a large role in the remodeling phase, secreting TGFβ1, IGF1, fibronectin, and activating fibroblasts to secrete collagen. M2 macrophages can also promote the differentiation and fusion of satellite cells.[71]

Formation of collagen fibers by fibroblasts begins with the transcription and translation of alpha peptides, which undergo multiple post-translational modifications inside the cell. The alpha peptides form a polypeptide chain, predominately made of the amino acids glycine and proline. An important step is the hydroxylation of proline and lysine residues on the alpha chains, which uses Vitamin C as a cofactor, forming hydroxyproline and hydroxylysine.[72] Subsequently, three alpha chains will form a triple helical structure in the endoplasmic reticulum, known as a procollagen. Different collagen types will have different makeups of alpha chains within its triple helix. For example, type I

collagen is made up of two α-1 chains, and one α-2 chain. Procollagen is then secreted outside of the fibroblast cell, where it becomes tropocollagen by the removal of its pro-peptide ends. As previously described, tropocollagen molecules form collagen fibers by covalent crosslinking.

In healthy tissue, the rate of collagen synthesis is matched by the rate of collagen degradation to maintain a proper microenvironment for muscle fibers. Degradation begins with the cleavage of collagen fibers by proteases known as MMPs. The exact process of collagen degradation continues to be studied and is quite fascinating. Because collagen molecules are so large, they cannot be cleaved whole, but need single alpha chains to be exposed for proteolysis. Importantly, MMPs contain both a hemopexin domain and a catalytic domain. The hemopexin domain binds and unwinds the collagen triple helical structure, allowing the catalytic domain to hydrolyze the separated collagen alpha chains. In addition to collagens, MMPs also degrade other ECM structures such as proteoglycans. Interestingly, the proteolysis of decorin by MMP-2, -3, or -7 can result in the release of TGF-β1, and therefore increase overall ECM accumulation.[73]

Tissue inhibitors of metalloproteinases (TIMPs) work to inhibit the actions of MMPs, further adding to the complexity of ECM turnover. Specific members of the TIMP family vary in their ability to inhibit specific members of the MMP family. Furthermore, the overall effect of TIMPs on ECM turnover is not straightforward, but is likely dependent on the type of tissue and health of that tissue.[74] For example, Kitamura et al. observed that under high glucose conditions, TIMP expression was increased and MMP expression suppressed in cultured glomerular mesangial cells,[75] suggesting hyperglycemia in diabetic patients contributes to renal pathology by an inhibition of ECM degradation. McLennan et al. further observed that connective tissue growth factor (CCN2 or CTGF), a known pro-fibrotic factor, mediates the inhibition of ECM degradation caused by hyperglycemia.[76] In these cases, increased TIMP-1 expression is associated with ECM accumulation due to the inhibition of proteolysis, which is commonly seen in various fibrotic tissues.[77,78,79] However, studies have also shown that genetic TIMP-1 deficiency in mice does not lead to a reduction of ECM accumulation

caused by several pathologic tissue models compared to wild-type mice, and can even worsen the condition.[80,81] In skeletal muscle, both MMPs and TIMPs are upregulated during wound healing. MMPs are thought to aid in the migration of satellite cells across the basement membrane by removing collagen obstructions.[82] There is some evidence that TIMPs help to control inflammation, which is likely why their complete removal is detrimental to wound healing.[81] Yet, as with other tissues, the ratio of TIMPs relative to MMPs in skeletal muscle pathologies is typically increased. Accordingly, there is some evidence that injection of MMP-1 into fibrotic skeletal muscle reduces overall collagen content.[83]

Fibrosis

In pathologic conditions of skeletal muscle, the formation of collagen and other ECM components accumulate excessively, resulting in fibrosis. Chronically fibrotic muscles generally exhibit increased stiffness and reduced force output compared to healthy muscle. Atrophy will likely accompany fibrosis and further contribute to the force loss. The cause of fibrotic progression in skeletal muscle can be from acute traumatic injuries, chronic overuse, or inherent genetic predispositions. In any case, it usually results secondary to an initial insult to the muscle and a failed regeneration attempt. Instead of satellite cell proliferation and fusion, there is a prolonged immune response that increases fibroblast activation and production of collagen. Once fibrotic programming has begun, it is difficult to slow or reverse the process due to a variety of factors.

For one, an increased area of ECM results in an increased distance from capillaries to myofibers, creating a hypoxic environment. A hypoxic environment increases expression of hypoxia-inducible factor-1α (HIF-1α) in muscle fibers. Coupled with increased TGF-β1 expression, HIF-1α stimulates production and secretion of CCN2/CTGF from muscle fibers.[84] Fibroblasts become activated by the secreted CCN2/CTGF, resulting in more ECM deposition, which further increases the distance of capillaries to myofibers. Valle-Tenney et al. have termed this process a “vicious oxygen cycle,” and propose it as a mechanism for fibrotic progression in skeletal muscle.[84]

In addition, prolonged inflammatory signaling results in oxidative stress due to the overwhelming presence of reactive oxygen species (ROS) relative to antioxidants. ROS generation is currently attributed to two main endogenous sources; mitochondria as a by-product of cellular respiration, and NADPH oxidase (NOX) as a primary function.[85] The NOX family includes seven isoforms, of which NOX2 and NOX4 are implicated in muscle regeneration due to their presence in phagocytes and fibroblasts, respectively.[86] ROS generated from these enzymes is a necessary step in the repair process by activating MAPK and NF-kB pathways, which helps to clear necrotic tissue and induce apoptosis of unwanted cells.[87] In a healthy regeneration model, the rise in ROS from NOX4 is combated by activation of Nrf2, a key regulator of antioxidant production.[88] Under quiescent conditions, Nrf2 is generally degraded by Keap1 (Kelch ECH associated protein 1), but upon stress Keap1 is inactivated and thus Nrf2 can translocate to the nucleus for transcription.[89] Nrf2 upregulates numerous antioxidant genes, including superoxide dismutase (SOD),[90] which converts superoxide (O_2^-) to hydrogen peroxide (H_2O_2).[85] The upregulation of SOD and other antioxidants are typically enough to neutralize excess ROS and restore the redox balance. Accordingly, the NOX4/Nrf2 ratio is emerging as a factor in the progression of multiple diseases, including idiopathic pulmonary fibrosis and cardiovascular disease[91,92]. Chronic ROS elevation under fibrotic conditions most likely augments the pathologic progression by stimulating TGF-β1 expression.[93] Furthermore, TGF-β1 and HIF-1α have been shown to increase NOX4 expression,[94,95] implicating hypoxia as a likely contributor of ROS production, creating a cyclic feedback loop. A recent study by Hanley et al. used pharmacological inhibition of NOX4 to prevent cancer-associated fibroblast accumulation and slow tumor growth *in vivo*.[96] Although therapies for reversing fibrosis will likely have to be multifaceted, NOX4 may be a good target of inhibition.

Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is genetic disorder caused by a mutation in the gene that encodes dystrophin, resulting in the loss of the protein throughout

various skeletal muscles.[97] Muscle fibrosis increases dramatically over time, as without dystrophin the muscles continually undergo damage and a subsequent failed regeneration leading to ECM accumulation and weakness. The exact mechanism by which the loss of dystrophin contributes to muscle degradation is not entirely understood, but it is well-known that the muscle is more susceptible to injury and has a reduced capacity to recover and regenerate. Eccentric muscle contractions have been shown to be particularly detrimental to patients with DMD, making exercise a cautious treatment option.[98,99] DMD affects males, and results in progressive loss of function leading to wheelchair assistance by early teenage years. Eventually the diaphragm weakens as well, causing respiratory failure; however, advancements in care have allowed many affected by the disease to live into their 30s.

A model for studying DMD was developed using mice that have lost expression of dystrophin, known as *mdx* mice. These mice exhibit a similar phenotype to DMD patients, although somewhat milder, making them a good model for learning more about the disease and fibrotic muscle in general. Similarly, golden retriever muscular dystrophy (GRMD) dogs are another good model and display a more severe phenotype closer to that of humans.[100] Muscle biopsies from DMD patients can also be used to understand disease progression; however, using animal models has the advantageous capability of testing experimental therapeutics.

Decades of research has revealed that skeletal muscle from DMD patients and animal models display an ever-increasing amount of ECM accumulation with age, along with a combination of abnormal hypertrophied and atrophied muscle fibers.[101,102,103] There appears to be early degradation of fast-twitch fibers (type IIx/b) resulting in greater proportions of slow-twitch (type I) fibers.[104] However, these slow-twitch fibers are also at a reduced oxidative capacity compared to healthy controls.[105] GRMD dogs were shown to have significantly increased CCN2/CTGF expression compared to control dogs just 30 days after birth, and increased collagen III expression at 60 days.[103] Furthermore, Stephens et al. observed increased relative amounts of collagen type III in DMD patients, along with thickened basement membranes of collagen IV surrounding muscle fibers.[102]

Goldpsink et al. suggest that age-related increases in fibrosis are not due to an increase collagen gene expression, but rather a reduction in degradation due to increased cross-linking of collagen fibers.[101] Additionally, specific ECM components may not proportionally increase with overall ECM accumulation, and can even decrease relative to age-matched controls. For example, Zanotti et al. found that decorin mRNA and protein levels were decreased in muscle biopsies of pediatric DMD patients relative to age matched controls, although significant fibrosis was present.[106] However, multiple studies have found results contradicting that notion,[107,108,109] possibly highlighting variability resulting from age and severity of phenotype.

Because of the debilitating condition DMD patients face, treatment options have been heavily researched, albeit with varying degrees of success. *Mdx* mice are typically the first to receive experimental treatments; however, because they exhibit a milder phenotype, treatments often work better in the mice than they do once in larger animals or clinical trials. Nonetheless, evaluating treatments with *mdx* mice is still an invaluable step in the process of finding something that works. Loss of myostatin, either from genetic manipulation or pharmacological inhibition, has shown promising results in *mdx* mice.[110,111] Results have included significant increases in muscle fiber size and maximal force output, as well as attenuation of fibrosis. Myostatin inhibition (by administration of ACE-031, a solubilized activin type IIB receptor) in a clinical trial of DMD patients saw positive results as well, although not as robust and accompanied by various side effects.[112] The avenue of myostatin inhibition for DMD patients looks promising, yet it does not solve the underlying issue of their genetic mutation. It can help relieve symptoms of the disease but will not cure it.

Potentially long-lasting therapeutic approaches for DMD include viral based gene therapies. Typical viral gene therapies take copies of the desired genetic information, as DNA or RNA, packed into a specific viral vector, such as adenovirus or lentivirus, which then enters the cell and releases the new genetic material. However, because the full-length dystrophin gene is so large (427 kD), it cannot fit inside a typical viral vector.[113] To solve this issue, shortened versions of

the dystrophin gene, or mini-dystrophin, have been developed that still result in functional proteins.[114] These can then be packaged into the viral vector of choice for systemic delivery of the new gene. Recent clinical trials from multiple drug companies using mini-dystrophin delivered via adeno-associated virus (AAV) capsids in boys with DMD have been encouraging enough to move on to their next phases. Moreover, AAV vectors have also been used to deliver CRISPR/Cas9 components in *mdx* mice to delete a specific mutated exon that is known to create a premature stop codon in the dystrophin RNA.[115] CRISPR/Cas9 technology likely has a promising future for DMD patients, leading to permanent dystrophin expression, in theory; however, the risk of off-target effects is a major limitation at the moment.[116]

Cerebral Palsy

Another condition presenting with impaired skeletal muscle health is cerebral palsy (CP). CP is thought to be caused by abnormal brain development or brain injury sometime during the prenatal, perinatal, or postnatal period.[117] The most common type of the disease is known as spastic CP, affecting the motor cortex region of the brain, which results in a reduced capacity to recruit motor units for voluntary movement. CP has been defined as a non-progressive condition,[118] yet muscle pathology secondary to the underlying condition can certainly become progressive.[119] Fibrosis is one of the hallmark signs of muscle pathology in patients with CP and likely contributes to the muscle stiffness observed. Patients with spastic CP often describe their muscles as feeling stiff when attempting to contract. Common complications of CP include scoliosis, hip dislocation and fixed muscle contractures.[119] Accordingly, gait problems often present an additional detriment to overall function and gait and motion analysis is frequently used to support treatment programs.[120] Exercise regimens for CP patients involving aerobic and/or resistance training, although safe, appear to be of little benefit in terms of gross motor function.[121,122]

Recent findings from children with CP indicate an increase in overall muscle collagen content compared to typically developing children.[123] Moreover, the ratio

of collagen I to III was decreased, suggesting an increased fraction of collagen III in the muscle of children with CP. Interestingly, proteoglycan analysis revealed an increase in decorin content but decrease in biglycan content. Biglycan has been shown to be important for maintaining synapse stability in the NMJ,[124] and there is reason to believe the NMJ is affected by CP.[125] Thus, the authors suggest administration of biglycan as a possible therapy for CP patients.[123]

Volumetric Muscle Loss

Volumetric muscle loss (VML) occurs when a significant portion of muscle is destroyed from a traumatic event, such as a high-energy blast wound during combat,[126] or from surgical debridement of tissue.[127] Thus, this condition is predominant among service members due to the risks of sustaining such a traumatic injury. Roughly 75% of all combat related injuries in the U.S. military from 2005 to 2009 were due to explosions, resulting in a high proportion of musculoskeletal injuries. Moreover, a cohort study of injured service members found that 92% of muscle conditions were identified as VML.[127] Because of the chronic nature of this condition with today's inadequate treatment options, many veterans of Operations Enduring Freedom, Iraqi Freedom, and New Dawn are dealing with lifelong functional disabilities due to VML. Although therapy is currently very limited regarding long term success, awareness of the issue is growing as evidenced by the increasing amount of research into the underlying physiology and treatment strategies.[128,129,130,131,132,133]

At present U.S. troops are involved in active combat in 14 different countries,[134] as the global war on terror continues to be fought. The amount of service members affected by VML will surely continue to rise as our presence in the Middle East and Africa remains. It is estimated that the global burden of traumatic injuries is upwards of $400 billion annually, and the average cost of disability payments for one VML patient over a lifetime is nearly half a million dollars.[127] Given that, discovering successful therapeutic methods to treat VML could have an enormous impact on the lives of currently disabled and newly injured service members both physically and financially.

In order to develop new therapies, it is imperative to first gain a better understanding of the pathophysiology responsible for the chronic disabilities observed. There have been great advances in this area recently, but still much to be discovered. For example, it is well known that following VML injury, the gap of tissue lost is too large for muscle fibers to bridge and thus muscle in the affected area is replaced by collagen deposition.[135,128] This fibrotic deposition extends into the remaining muscle tissue, causing a loss of force output, reduced range of motion and increased stiffness.[130,136] **Figure 5** depicts the temporal change of various markers in a rat model of VML injury. The graph was adapted from primary data from Corona et al. and Aguilar et al., in addition to preliminary data on innervation from the Greising lab.[136,137] Strength and fiber numbers are drastically reduced early on, followed by a partial recovery around 14 days and subsequent plateau or further decline. The strength loss remains roughly 50% and never fully recovers. Innervation follows a similar trend and continues to decline at least through 48 days after injury. Furthermore, recent work examining muscle architecture has shown that muscle fiber lengths are reduced after injury, as expected; however, the physiological cross-sectional area (muscle volume/fiber length) is preserved, which was an unexpected finding.[138] The authors suggest that this preserves potential maximal force producing capacity.

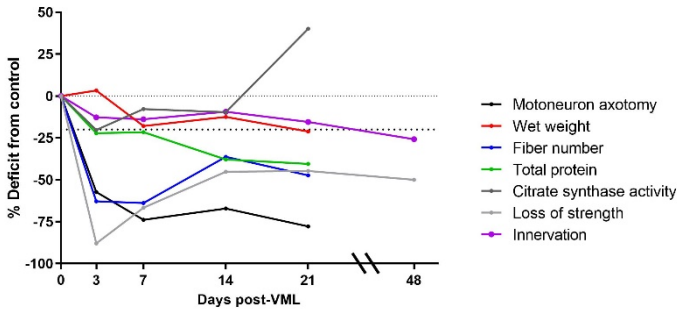

Figure 5. Post-VML deficits relative to controls. Modified from Corona et al. 2013, Aguilar et al. 2018, and Sorensen et al. in preparation.

Changes within the ECM after VML injury have not been studied as extensively to this point. It has been shown that there is a dramatic increase in total collagen content and that gene expression of various pro-fibrotic factors (TGF-β1, CTGF, etc.) are increased.[133] Therefore, an increase in overall collagen content following VML injury is expected to be observed; however, the organization of collagen in

terms of isoform proportions and how they may change over time is not understood. Advancing our knowledge in this aspect may be a big step in producing and evaluating new anti-fibrotic treatments that could amplify rehabilitation methods.

Pharmacological targets

Current treatment options for VML include filling the lost muscle with various versions of decellularized collagen scaffolds and/or minced muscle grafts, with the hopes of guiding muscle regeneration.[128,139,140,141] Although some studies using animal models have seen beneficial effects using these methods, no results to date have been clinically significant in terms of restoring muscle function. Aside from refining scaffold implantation, pharmacological inhibition or overexpression of various proteins has the potential to aid current treatment options. In particular, anti-fibrotic drugs may have the ability to allow scaffolds and muscle grafts to work better, by reducing the ECM pathology. To date, drugs that inhibit TGF-β1 activity have been the most studied type of anti-fibrotic, due to its well-known role in ECM accumulation.

Losartan is an FDA-approved anti-hypertensive drug that indirectly blocks TGF-β1 by inhibiting angiotensin type II receptor I (AT1), and therefore has potential anti-fibrotic effects. AT1 aids in fibrotic signaling by activating the Smad2/3 pathway, which increases production of TGF-β1 and collagen.[142] By inhibiting AT1, Losartan was shown to reduce fibrosis in rats after VML injury; however, it also led to a reduction of maximal force production.[133] The force loss is likely due to the fact that the fibrotic deposition has capacity to transmit force,[136] and reduction of this without concurrent myogenic increases will hinder force output. However, there is good rational to combine this treatment with a subsequent myogenic treatment to overcome the limitation.

Nintedanib is another anti-fibrotic drug that has shown effectiveness in reducing fibrosis after VML injury.[132] Nintedanib is FDA-approved for the treatment of idiopathic pulmonary fibrosis (IPF).[143] It is a tyrosine-kinase inhibitor, and therefore targets fibroblast growth factor receptor, platelet-derived growth factor receptor,

and vascular endothelial growth factor receptor, which are known pro-fibrotic factors.[144] Regardless of the tissue affected, fibrotic pathologies generally have similar signaling pathways, and thus Nintedanib has been evaluated in the treatment of various diseases aside from IPF.[145,146,147] Similar to findings with Losartan, recent results using Nintedanib in a porcine model of VML injury demonstrated reduction of fibrosis, which led to overall force loss.[132] This further corroborates the notion that fibrotic tissue in skeletal muscle plays a role in force transmission. Yet any hope of returning muscle function to normal after VML would likely have to take this seemingly backwards step in order to return the ECM to a more permissive state for myogenesis. Conversely, Nintedanib treatment in *mdx* mice resulted in reduction of fibrosis with improved muscle function measurements as recorded by electromyography.[148] The difference allowing *mdx* mice to improve muscle function is presumably the fact that the fibrosis is spread throughout the muscle with viable satellite cells able to increase myogenesis once the fibrosis is reduced. After VML injury, satellite cells in the defect area are removed and thus it becomes a challenge to replace the defect with anything other than ECM deposition.

A newer emerging anti-fibrotic medication also designed for IPF, known as Pamrevlumab, has shown encouraging early results.[149] Pamrevlumab is a monoclonal antibody that inhibits CCN2/CTGF activity, a factor involved in fibrosis as previously discussed. The drug is currently in phase 3 clinical trials for IPF and locally advanced unresectable pancreatic cancer, as well as phase 2 trials for DMD and COVID-19. Results from Phase 2 clinical trials of IPF patients suggest Pamrevlumab is arguably more effective at treating the condition than Nintedanib and had fewer adverse effects.[149] Furthermore, Pamrevlumab treatment in *mdx* mice reduced fibrosis, increased muscle strength, and improved satellite cell grafting ability.[150] Notably, these changes were achieved without any significant changes in TGF-β1 signaling, suggesting that CCN2/CTGF acts downstream of TGF-β1 and can independently modulate the fibrotic response. Pamrevlumab has yet to be evaluated in a model of VML injury.

The major gaps in the current understanding of fibrosis following VML injury involve the sequential cellular and molecular remodeling that results in a state unconducive to myogenic treatments. Protein quantification of specific collagen isoforms or proteoglycans following VML injury has yet to be observed. Similarly, protein quantification of pro-fibrotic factors, such as CCN2/CTGF, has not been measured over time. Furthermore, observations relating to fibrosis after VML injury have largely been focused on the defect area and not the remaining uninjured muscle. These measurements would be advantageous in deducing the optimal timing for future treatments, and potentially finding new targets. To address the important questions raised above, my thesis had the following specific aims and hypotheses:

Specific Aim 1: Evaluate temporal changes in collagen isoform proportions and orientation of collagen fibers within the remaining muscle after VML injury.

Hypothesis 1: Collagen III will be increased relative to collagen I at all time points. There will be a shift in more densely packed collagen with increasing time post-VML injury.

Specific Aim 2: Evaluate CCN2/CTGF signaling in defect area and remaining muscle following VML injury.

Hypothesis 2: CCN2/CTGF will be significantly increased in the defect area following VML injury.

Specific Aim 3: Evaluate temporal changes in decorin levels relative to overall collagen content after VML injury.

Hypothesis 3: Absolute decorin levels will increase after VML injury but will be reduced relative to total collagen content.

These specific aims and hypotheses are addresses in Chapter 2, which include the final manuscript of my research project (in preparation for submission to *Connective Tissue Research*). Finally, Chapter 3 outlines future directions for this work.

Chapter 2: Research Project

Overview

Purpose/Aim: Volumetric muscle loss (VML) is a devastating orthopaedic injury that results in chronic persistent functional deficits, loss of joint range of motion, pathologic fibrotic deposition and lifelong disability. However, to date there is only limited mechanistic understanding of the VML-induced fibrosis. We examined the temporal changes in the fibrotic deposition early after VML injury to identify a potential window of effective intervention. **Materials and Methods:** Adult male Lewis rats (n=48) underwent a full thickness ~20% (~85mg) VML injury to the tibialis anterior (TA) muscle and were harvested for genetic and histologic evaluation. In order to temporally track fibrotic changes, we evaluated extracellular proteins at 3, 7, 14, 28, and 48 days post VML-injury both biochemically and histologically. The contralateral TA muscle served as the control group. **Results:** The ratio of collagen I/III was decreased at 3, 7 and 14 days post-VML injury, but significantly increased at 48 days post injury. Decorin content followed an opposite trend, significantly increasing by day 14 before dropping to below control levels by 48 days. Histological evaluation of the defect area indicates a shift from loosely packed collagen at early time points post-VML, to a densely packed fibrotic scar by 48 days. CCN2/CTGF signaling was significantly increased throughout the muscle at every time point. **Conclusions:** The shift from early wound healing efforts to a predominately fibrotic muscle tissue appears to occur rather synchronously via the significant reduction of collagen III and increase in collagen I after 21 days post-VML. This observation is supported by a dramatic increase in densely packed collagen after 21 days post-VML. Thus, there appears to be an early window for effective anti-fibrotic intervention, potentially by targeting CCN2/CTGF or using decorin as a therapeutic.

INTRODUCTION

Pathologic fibrosis is a common sequela of severe injuries and diseases affecting various tissues, including skeletal muscle. It is characterized by an excessive accumulation of extracellular matrix (ECM) components, namely collagen, and in skeletal muscle generally seen in conjunction with atrophy.[64] The combination of fibrosis and atrophy limits muscle function by reducing range of motion and maximal force production, effectively reducing the overall quality of life for those affected. In minor acute injuries to skeletal muscle, such as strains and contusions, there is a transient period of inflammation and ECM remodeling, which is ultimately resolved and replaced by repaired and/or regenerated skeletal muscle fibers.[71] However, in more severe pathologies there appears to be a tipping point where muscle regeneration is repressed, and ECM deposition progresses uninhibited.[151]

A critical component of the fibrotic repair process is the timely activation and balance of pro-inflammatory M1, and anti-inflammatory M2 macrophages. M1 macrophages are proposed to be responsible for clearing necrotic debris and activating satellite cells, while M2 macrophages control matrix remodeling and fusion of satellite cells.[71] Notably, M1 and M2 macrophages exist at the ends of a continuum sequence of possible states without clear boundaries, and typically transition in the direction from M1 to M2.[152] The imbalance or prolonged activation of either end of the continuum will likely result in detrimental effects to the tissue. For example, the initial inflammatory response is crucial for tumor necrosis factor (TNF) mediated apoptosis of fibro/adipogenic progenitors (FAPs), a major source of ECM producing fibroblasts.[153] However, too early of a transition to an anti-inflammatory state increases transforming growth factor-β1 (TGF-β1) expression, resulting in decreased TNF-induced FAP apoptosis and thus fibroblast accumulation.[153] Overexpression of TGF-β1 further contributes to fibrotic development by stimulating fibroblasts and regenerating myofibers to produce collagen.[154] As the area of ECM increases, so does the distance from muscle fibers to capillaries, resulting in a hypoxic local environment. In fact, hypoxia coupled with TGF-β1 expression has recently been found to stimulate myofiber secretion of

connective tissue growth factor (CCN2/CTGF), further increasing fibrosis and creating a cycle of progressive fibrotic development.[84]

While research emphasis of muscle pathologies has been on the promotion of muscle fiber growth and regeneration without regard to fibrosis, it is becoming more recognized that the microenvironment in which muscle fibers reside is crucial to successful regeneration. The composition of ECM layers in healthy skeletal muscle is fairly well understood,[155] yet how the ECM becomes altered from various fibrotic pathologies is not as clear. Therefore, a better understanding of the skeletal muscle microenvironment in specific pathologic conditions is necessary to increase the probability of improving regeneration and functional outcomes. Collagen types I and III are the most abundant fibrillar isoforms in skeletal muscle ECM, with the perimysium and epimysium layers predominately composed of type I.[156] Type III collagen appears to be more abundant in the endomysium layer, yet still a component of all layers.[156] Type IV collagen is the most abundant non-fibrillar isoform, primarily making up the basement membrane of muscle fibers, while type VI collagen provides a link from the basement membrane to the ECM.[157] Recent work has indicated that type VI collagen is vital for maintaining satellite cell viability in skeletal muscle.[158] Moreover, proteoglycans residing within the ECM, namely decorin and biglycan, play an important role in regulating collagen fibril formation. Genetic deficiencies in either decorin or biglycan have been shown to cause structural abnormalities in collagen fibers. [159,67]

Conditions presenting with known pathologic fibrosis of skeletal muscle include Duchenne muscular dystrophy (DMD), cerebral palsy (CP), and polymyositis (PM). Interestingly, though they differ in etiology (genetic, congenital, and autoimmune, respectively), all three conditions display fairly similar fibrotic characteristics. Biopsies from DMD, CP, and PM patients are observed to have an increase in the proportion of collagen III in muscle ECM.[102,123,160] Recently, Smith et al. revealed an increase in decorin content but decrease in biglycan content in patients with CP.[123] As the authors suggest, this is plausibly associated with abnormal neuromuscular junctions found in CP,[125] as biglycan plays a role in maintaining synapse stability.[124] Yet, findings in DMD patients have not been as

clear, with some groups reporting decreases in decorin and biglycan mRNA, [106,161] although most have found signifcant increases in mRNA and protein content of both.[107,108,109]

Volumetric muscle loss (VML), defined as the “traumatic or surgical loss of skeletal muscle with resultant functional impairment”,[126] similarly generates a robust and lasting fibrotic response but is less understood. As VML is the result of an acute traumatic injury rather than a chronic underlying condition, the fibrosis observed after injury may display temporal differences compared to the aforementioned conditions. Clinically, two major problems exist in the care of VML injuries. First, no standard of care has been established to address the loss of function and mass of skeletal muscle. Second, the pathologic fibrotic response impedes muscle healing and regeneration, causing destruction of muscle architecture and altering the microenvironment of the muscle. At the tissue level, it is clear the lost skeletal muscle is incapable of endogenously regenerating and the defect area is replaced by an aberrant overproduction of ECM accumulation.[128] Yet at the molecular level, there is limited understanding of the makeup of the resulting fibrotic mass in terms of collagen proportions and organization, which could be transformative to develop and evaluate anti-fibrotic treatments for skeletal muscle. Previous evaluation of VML-injured muscle has indicated an overall increase in collagen content and upregulation of fibrotic genes such as TGF-β1, CCN2/CTGF, and various collagen isoforms (e.g., types I and III);[133,131,130] however, understanding of the true content and ratio of these proteins over time, as determined biochemically, is lacking. Herein the temporal fibrotic response after VML injury was evaluated in a rat model of VML injury to test the hypothesis that ECM components become proportionally altered after injury compared to healthy muscle.

MATERIALS AND METHODS

Ethics approval

All protocols were approved by the Institutional Animal Care and Use Committee at the University of Minnesota; in compliance with the Animal Welfare Act, the

Implementing Animal Welfare Regulations and in accordance with the principles of the Guide for the Care and Use of Laboratory Animals.

Experimental design and animal model

Male Lewis rats (Charles River, n=39) underwent unilateral VML injury to the left tibialis anterior (TA) muscle and were randomized to terminal groups of 3, 7, 14, 21, or 48 days post-VML. TA muscles were harvested from injured (left) and contralateral (right) hindlimbs at the given terminal time points for histological and biochemical analysis. Muscles from the contralateral limbs were used as controls for all outcome measures and collapsed into one group. Rats were given at least one week to acclimate to the facility prior to study. All rats were given food and water ad-libitum throughout the duration of the study and maintained on a 14:10 hour dark:light cycle. At the time of surgery and randomization, rats were on average at 3.7 ± 0.2 months of age and 379 ± 21g of weight. At the terminal time point, rats were euthanized by overdose of sodium pentobarbital (>100mg/kg i.p.) while under isoflurane anesthesia and skeletal muscle was harvested and stored for further analysis.

Surgical creation of volumetric muscle loss injury

All rats in the study underwent surgery to create a full thickness VML injury to the left TA muscle, as previously described.[162] Rats received analgesic injection (SR buprenorphine, 1.2mg/kg s.q.) roughly 2 hours prior to the start of surgery. Rats were then anesthetized with isoflurane (1.5-3.0%) throughout the duration of the surgery. Briefly, an anterior incision was made through the skin, and the fascia was cut to expose the TA muscle. A metal plate was inserted behind the TA, and a 6mm punch biopsy was taken to remove ~20% (85 ± 13mg) of the TA muscle. Proline sutures were used as a reference to identify the defect area at the time of harvest. Following VML injury, the fascia was sutured and the skin was stapled. Rats were monitored twice daily for three days post-surgery, and once a week thereafter for signs of pain and complications. There were no incidence of adverse

events in the study and rats healed and recovered ambulatory ability promptly after surgery.

Histological and image analyses

For each terminal time point, the whole TA muscle from both left and right hindlimbs was harvested, blotted dry and subsequently weighed. The distal half of the VML defect area and approximate area on the contralateral limb was mounted on cork using tragacanth gum for histological evaluation. The tissue was placed in 2-methylbutane cooled by liquid nitrogen to freeze, and subsequently stored at -80°C until further analysis. The TA muscle was sectioned at 10μm using a Leica cryostat and microtome and placed on glass slides.

Muscle fiber numbers and area of fibrosis were obtained using a Masson's trichome stain (Thermo Scientific Richard-Allan Scientific Chromaview Kit; 87019) following manufacturer instructions. Once stained, images were obtained using a 20X objective (0.75 NA, 0.5 μm/pixel resolution) on the brightfield Huron TissueScope LE slide scanner (Huron Digital Pathology, St. Jacobs, ON, Canada). Images were then analyzed using FIJI (ImageJ) software,[163] with the "multi-point" function used to count fibers and the "freehand selection" function used to measure area of fibrosis.

Visualization and quantification of collagen orientation was obtained using a picrosirius red stain under polarized light. Briefly, frozen sections were fixed in 4% PFA for 10 minutes, rinsed in deionized water and subsequently placed in picrosirius red solution (0.1% Direct Red 80, 1.2% picric acid; Abcam, ab246832) for 60 minutes. Sections were then quickly rinsed in 3 changes of 0.5% acetic acid (~10 dips each), dehydrated in 2 changes each of 95% and 100% alcohol (1 minute each) and cleared in xylenes before being immediately mounted with DPX mounting medium (Electron Microscopy Sciences). The slides were then viewed and imaged using a Nikon Eclipse 200 light microscope with diascopic cross-polarized filter at 40x magnification (E Plan 40x/0.65 OFN20) using digital camera interfaced to Nikon Elements D software. Regions of interest (ROI) for analysis included the VML defect area, the remaining uninjured muscle, and two images

along the border region of the defect and remaining muscle. For the contralateral control limbs, analogous regions were imaged, however a single control value is presented for comparison. During all imaging gain, offset, gamma, and exposure were constant. The area of red and green staining within ROI boxes were obtained using FIJI.[163] Specifically, two thresholds were applied using the '*a* channel*' in the CIELAB color space. A green threshold was made by setting the upper threshold to 128 and lower threshold to 0, while adjusting the lightness value to ~30. The upper threshold in the '*b* channel*' was set to 220 in order to remove yellow pixels. An orange/red threshold was then made by setting the '*a* channel*' lower threshold to 128 and upper threshold to 255, while adjusting the lightness value to ~45. A yellow threshold was added to the calculations of orange/red by setting the upper threshold of the '*a* channel*' to 128, and lower threshold of the '*b* channel*' to 220. The images were then binarized in their respective thresholds and measured for area. Data is presented in two ways, first, as the orange/red/yellow and green area independently and second, as a ratio of the orange/red/yellow to green area. With a lower ratio indicating collagen that is aligning in parallel to muscle fibers and greater ratios indicating disorganized collagen alignment to the longitudinal axis of the muscle fibers as previously described.

The TA was also stained with anti-CCN2/CTGF. Following fixation in 4% PFA for 10 minutes, sections were washed with PBS and blocked in 10% goat serum in PBS for 1 hour. Sections were incubated in primary antibody solution (5% goat serum in 0.5% Tween-TBS) overnight in 4°C. Primary antibodies included anti-CCN2/CTGF (Abcam: ab5097, 1:200). Sections were washed with TBS and incubated with an Alexa-Fluor 488 conjugated secondary antibody in 5% goat serum in 0.5% Tween-TBS for 1 hour at room temperature. DAPI was also added to stain for nuclei. Sections were washed with PBS and mounted with Fluoro-Gel mounting medium (Electron Microscopy Sciences). Imaging and analysis of muscle ROI's followed the same strategy as described for the picrosirius red. Briefly, image ROI's were collected within the VML defect, the remaining tissue and at the border where the VML defect and the remaining tissue converge. For the control limbs, analogous regions were imaged and a single control value is

presented for comparison. Images were taken using an automated Nikon C2 upright confocal microscope (Nikon Instruments Inc., Melville, NY) at a magnification of 20X (PlanApo 0.75 NA, 0.31 µm/pixel resolution). For analysis, images were binarized and the image intensity threshold was determined using the Otsu algorithm (FIJI) to measure the CCN2/CTGF area per ROI. Data are presented as a percentage of the total image area.

In all cases, the expected staining patterns in normal skeletal muscle were observed and the specificity of anti-labeling (CCN2/CTGF) was confirmed by the absence of staining outside expected structures and was consistent with manufacturer's technical information. For display purposes only, images were down-converted, without introducing any changes in brightness or contrast and produced in Adobe Photoshop (Adobe Systems Inc.). During all fluorescent imaging, the laser intensity and gain were kept consistent across samples. Investigators were blinded during all imaging and post-imaging analyses.

Biochemical analyses

Following harvest, the uninjured portion of TA muscle tissue distal to the VML defect area, and approximate area on the contralateral limb, was cut vertically into two pieces (medial/lateral), snap-frozen using liquid nitrogen and stored at -80^{o}C until analysis. The lateral piece of each muscle was used to measure specific collagen isoforms, in addition to the protein content of decorin. Quantification of collagen I, III, IV, and VI (MyBioSource, COL1α2-MBS2023826, COL3α1-MBS2023591, COL4α1-MBS7226846, COL6α1-MBS102349) was conducted by ELISA. Briefly, tissues were weighed and homogenized in a 10-mM phosphate buffer at a ratio of 1:100 (mg/µl), using a glass pestle tissue grinder over ice. The resulting homogenate was centrifuged for 5 minutes at 10,000 *g*, and the supernatant was aliquoted for immediate storage at -80°C. Total protein content was analyzed using the Protein A280 application (1 Abs = 1 mg/ml) on a NanoDrop One spectrophotometer (Thermo Scientific) after one freeze/thaw cycle. Samples were analyzed for specific collagen isoforms using the respective ELISA kits as indicated by the manufacturer and normalized to protein content. The distal-medial

piece of each TA muscle was then used to measure total collagen content. This was determined by quantifying the content of hydroxyproline in the muscle sample, as previously described.[164]

Immunoblot analyses

Fifteen µg of total protein were separated by 4-20% SDS-PAGE, transferred onto a PVDF membrane, and immunoblotted. Anti-decorin (Proteintech: 14667-1-AP; 1:500) primary antibody was detected using a corresponding horseradish peroxidase-conjugated secondary antibody (Cell Signaling: #7074, 1:1000). Protein detection was obtained using Clarity Max ECL Western Blotting Substrate (Bio-Rad). Immunoblots were visualized with stain-free and chemiluminescent imaging using a ChemiDoc System (Bio-Rad Laboratories, Hercules, CA). Immunoblots were normalized to total protein in lane and quantified using Bio-Rad Laboratories Image Lab software (Hercules, CA). For decorin primary antibody, single bands were identified at 70 kDa as expected and consistent with manufacturer's technical information.

Statistical analysis

Analysis was performed with JMP Pro statistical software (version 14.2 SAS Institute, Cary, NC, USA). Dependent variables for controls, day 3, 7, 14, 21, and 48 were analyzed by one-way ANOVA across time points. When significance was found, Tukey's HSD post-hoc analysis was used to ascertain specific time point significance. Data are presented as mean±standard deviation (SD). Statistical significance level was set at $p \leq 0.05$. During all evaluation the research team was blinded to the experimental groups.

RESULTS

Animals

Following VML surgery, all rats recovered promptly and without complications. Overall body weights steadily increased with time after injury, as expected. Rats in the 48 day group were on average 15% heavier at the terminal time point than at

surgery, as expected. In parallel the weight of the control (right, uninjured) TA muscle increased over time post-injury. While the weight of the VML-injured TA muscle decreased compared to control at all times points after day 3. Specifically, at 48 days post-VML, the injured TA muscle was ~18% smaller than the control TA muscle (Table 1).

Histologic Presentation of Fibrosis following VML Injury

Table 1. Whole body and skeletal muscle characteristics

		Days post-VML					
	Contralateral control (n=9)	**3 (n=8)**	**7 (n=7)**	**14 (n=8)**	**21 (n=8)**	**48 (n=8)**	**p-value**
Surgical body weight (g)	- -	383.4 ± 26.1	377.0 ± 21.5	374.4 ± 12.3	394.8 ± 22.5	385.6 ± 12.1	0.291
Terminal body weight (g)	- -	368.8 ± 22.2 §‡	368.7 ± 22.5 §‡	376.3 ± 19.0 §‡	406.9 ± 26.4 §	444.6 ± 12.5	<0.0001
Injured TA weight (mg)	- -	689.1 ± 83.5 †	616.9 ± 85.0	582.5 ± 37.4	601.0 ± 65.9	599.1 ± 80.4	0.049
Contralateral TA weight (mg)	690.9 ± 67.6	639.0 ± 33.1 §‡	645.2 ± 75.5 §	704.9 ± 32.4	719.6 ± 27.1	730.5 ± 23.4	0.001
TA/body weight (mg/g)	1.73 ± 0.11 §	1.88 ± 0.31 §‡†	1.67 ± 0.18 §	1.55 ± 0.03	1.49 ± 0.22	1.35 ± 0.17	<0.0001
TA protein content (µg/mg)	93.44 ± 15.56	80.74 ± 8.18 §	68.50 ± 13.93 *	73.12 ± 11.90 *§	72.89 ± 5.60 *§	54.26 ± 9.99 *	<0.0001

Contralateral controls from a sub-set of animals are collapsed over time points for data from TA muscle.
Data analyzed by one-way ANOVA; differences are indicated as *different than control; †different than day 14; ‡different than day 21; and §different than day 48.

Gross histologic presentation of the TA muscle following VML injury was qualitatively evaluated using Masson's Trichrome staining (Figure 6). Broadly there is significant fibrotic deposition that begins to develop as early as 7 days post-VML, as well as substantial cellular infiltration into the VML defect area. By 48 days post-VML the fibrosis appears to spread more prominently into the remaining muscle.

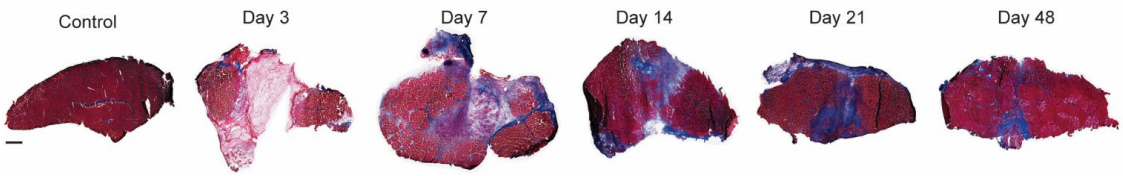

Figure 6. Masson's trichrome staining was conducted across time points to evaluate the progressive fibrotic deposition following VML injury. Representative images from each time point are displayed, connective tissue is blue and muscle fibers are red. Scale 1mm.

Staining for picrosirius red was used to visualize and evaluate collagen packing (Figure 7A-B). Using cross-polarized filters, staining exploits the birefringent properties of collagen, displaying a spectrum of red to green hues and eliminating non-collagenous structures. Historically, hue differences were thought to distinguish collagen I from collagen III based on thickness, with collagen I (thicker) staining red/orange/yellow and collagen III (thinner) staining green.[165] However, it is unlikely hue differences are specific to collagen types, given that immature collagen I may be relatively thin. Rather, it is likely indicative of collagen packing, whereby loosely packed collagen appears green and densely packed collagen appears red/orange/yellow. Therefore, the area fractions of collagen stained red/orange/yellow and the area stained green were quantified in the TA muscles. Muscle sections were evaluated at three systematically selected ROIs: the remaining muscle, the defect area, and the border between the defect and remaining muscle. As expected, the total fraction of the polarized area increased over time in the defect and border regions post-VML. Specifically, the green area fraction increased significantly at 7, 14, and 21 days in both regions before declining at 48 days ($p \leq 0.045$), while the red/orange/yellow fraction continued to significantly increase through 48 days ($p \leq 0.0001$; Figure 7C-E). Thus, the ratio of red/orange/yellow to green in defect and border regions was decreased at early time points post-VML before significantly increasing after 48 days ($p \leq 0.007$; Figure 7G-H). Remarkably, the total fraction area in the remaining muscle was also significantly increased after 7 days post-VML. While the red/orange/yellow area was significantly increased at all time points after 7 days, the green area was significantly increased at 21 days post-VML. The ratio of red/orange/yellow to green in the remaining muscle was slightly lower than controls following VML, and

significantly so at 3 and 21 days post-VML (p≤0.018; Figure 7F), indicating an increased relative fraction of green, or loosely packed collagen.

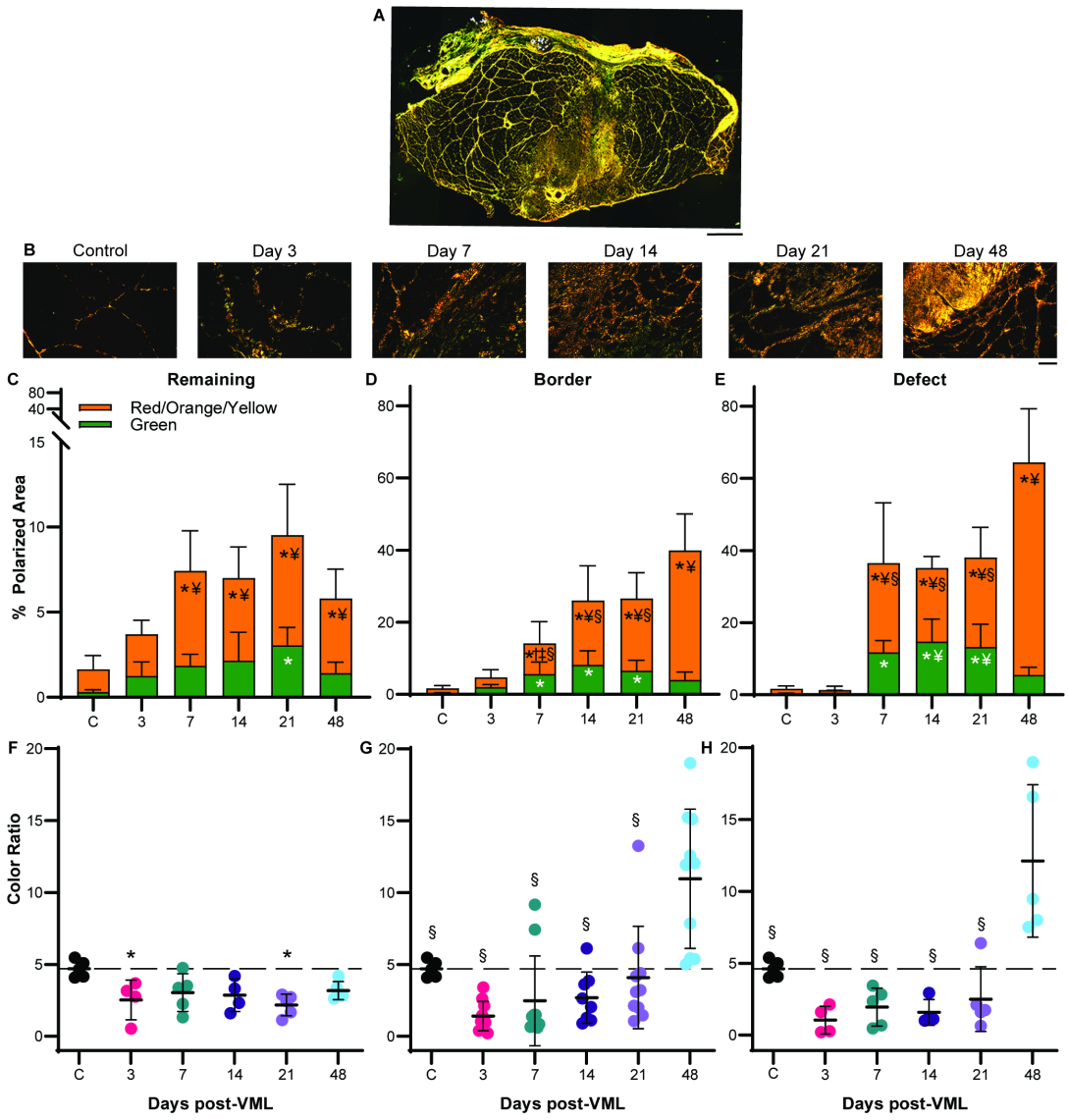

Figure 7. **A)** Representative whole TA muscle stained with picrosirius red; example is from 21 days post-VML. Areas staining green represent loosely packed collagen, while areas staining red/orange/yellow represent more densely packed collagen. Scale 1mm. **B)** Representative images taken from the border region of defect area across time points and analogous control region. Scale 50µm. Total area fraction of collagen separated into red/orange/yellow and green fractions across post-VML time points are shown for the **C)** remaining muscle ($p<0.0001$), **D)** border region ($p<0.0001$), and **E)** defect area ($p<0.0001$). Ratio of orange/red/yellow fraction to green fraction are plotted from **F)** remaining muscle ($p<0.009$), **G)** border region ($p<0.0001$), and **H)** defect area ($p<0.0001$). Dashed line indicates averaged control average. Images were randomly selected from respective regions for analysis. One image was analyzed from remaining muscle and defect area, while two images were analyzed from each border region and plotted as separate points. Data analyzed by one-way ANOVA and indicates as *different than control; ¥ different than day 3; †different than day 14; ‡different than day 21; §different than day 48

Staining for CCN2/CTGF was used to observe and evaluate fibrotic signaling in the same ROIs (Figure 8A-B). The percentage of area positive for CCN2/CTGF was significantly increased ($p \leq 0.004$) as early as 3 days post-VML throughout the muscle and defect, and further stabilized or increased through 48 days post-VML. In the defect region, the CCN2/CTGF positive area was 1.4-fold greater from 3 to 7 days post-VML. Similar increases were seen in the border region. Notably, throughout the remaining muscle CCN2/CTGF positive area did not increase further at any time point after 3 days post-VML but maintained significantly increased above control levels.

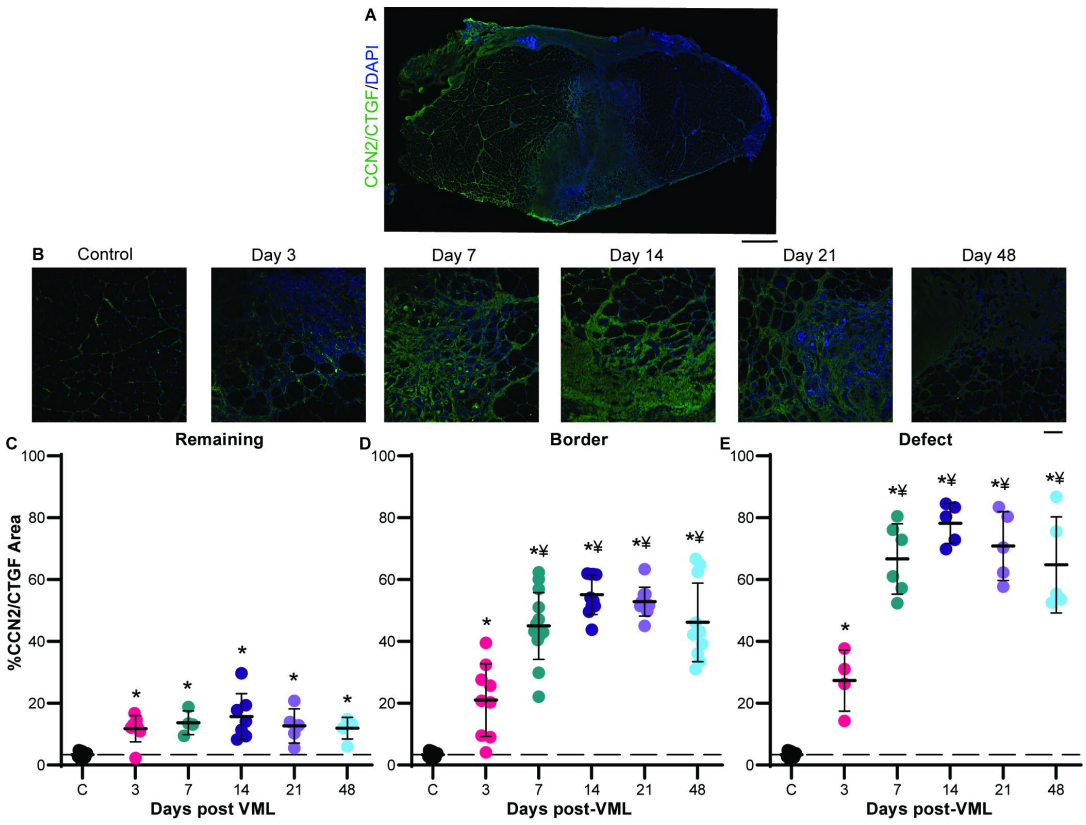

Figure 8. A) Representative whole TA muscle stained for CCN2/CTGF (Green) and DAPI (Blue); example is from 21 days post-VML. Scale 1mm. **B)** Representative images taken from the border region of defect area across time points and analogous control region. Scale 50µm. Percent area positive for CCN2/CTGF across post-VML time points are plotted from **C)** remaining muscle ($p<0.0001$), **D)** border region ($p<0.0001$), and **E)** defect area ($p<0.0001$). Dashed line indicates averaged control area. Images were randomly selected from respective regions for analysis. One image was analyzed from remaining muscle and defect area, while two images were analyzed from each border region and plotted as separate points. Data analyzed by one-way ANOVA and indicates *=different than control; ¥ different than day 3

Variations in Collagen, Isoforms, and Signaling following VML Injury

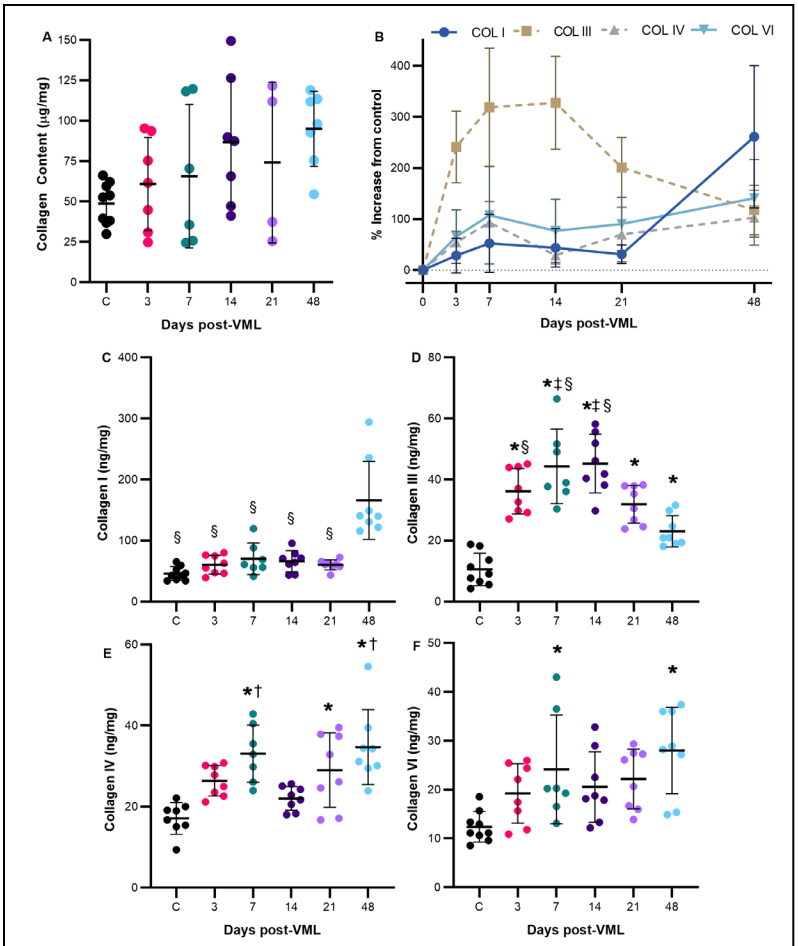

Figure 9. A) Total collagen content from injured TA normalized to muscle size (p=0.09). **B)** Collagen isoform temporal percent increase from control, 0% indicates control levels. Protein content of **C)** collagen I (p<0.0001), **D)** collagen III (p<0.0001), **E)** collagen IV (p<0.0001), and **F)** collagen VI (p=0.003), normalized to total protein content of the sample are plotted across time points post-VML. Data analyzed by one-way ANOVA and indicates *different than control; ¥ different than day 3; †different than day 14; ‡different than day 21; §different than day 48

Although there was an upward trend of total collagen, as determined by hydroxyproline content, it was not significantly increased at any time post-VML in the remaining muscle (p=0.09; Figure 9A), likely due to unexpected sample variability. However, there was a trend for the collagen content at 48 days post-VML to be about 1-fold greater than the control.

Protein levels of collagen III (normalized to protein content) were significantly increased at all time points following VML injury compared to control (p=0.026; Figure 9D). At 3 and 14 days post-VML, collagen III was increased by 2 to 3-fold, respectively, before a gradual decrease in protein levels by day 48. Conversely, collagen I was not significantly increased until 48 days post-VML, where it rose ~2.5-fold compared to control (p<0.0001; Figure 9C). Accordingly, the collagen I:III ratio was reduced at 3, 7, 14, and 21 days post-VML, yet increased at 48 days. Protein levels of collagen IV and VI were both significantly increased at 7 and 48 days post-VML (p≤0.031; Figure 9E-F), yet scaled rather proportionally to the overall increases in total collagen.

Content of decorin within the remaining muscle was significantly increased at 14 days post-VML compared to control (p=0.043; Figure 10A). However, by 48 days post-VML decorin content was slightly lower than control and significantly reduced compared to day 3 and day 14 (p≤0.036).

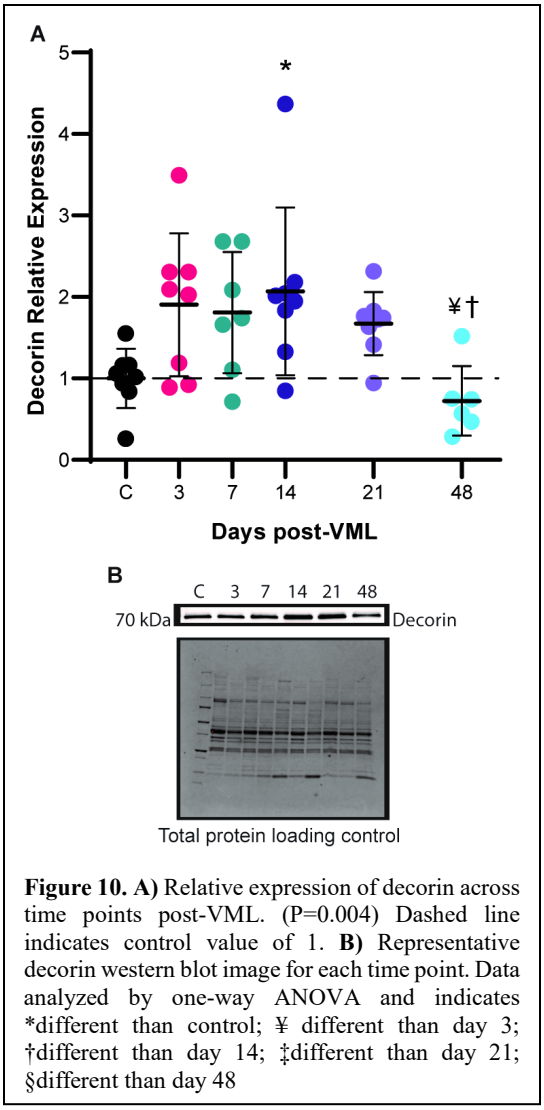

Figure 10. A) Relative expression of decorin across time points post-VML. (P=0.004) Dashed line indicates control value of 1. **B)** Representative decorin western blot image for each time point. Data analyzed by one-way ANOVA and indicates *different than control; ¥ different than day 3; †different than day 14; ‡different than day 21; §different than day 48

DISCUSSION

Pathologic fibrosis is detrimental to muscle tissue by increasing stiffness and reducing maximal force production, in addition to impeding muscle regeneration by inhibiting satellite cell migration and differentiation[166,167]. However, the implications of fibrotic progression after VML injury on the overall goal of restoring muscle function are often overlooked. Previous animal models of VML injury have established a general increase in collagen I and III as well as pro-fibrotic factors observed via histological and transcriptome data.[131,130] Yet, a detailed temporal response of ECM proteins and collagen organization post-VML injury has not been previously observed. Herein VML injury is shown to significantly increase the proportion of collagen I throughout the remaining muscle and densely packed fibrotic scar tissue in the defect region 48 days post-VML. Interestingly, decorin, a known inhibitor of fibrosis, appears to have a somewhat inverse relationship with collagen I after injury, whereby it was significantly increased at 14 days before decreasing by 48 days post-VML to levels slightly lower than control muscle. However, before remodeling to this non-permissive state, the proportion of collagen III and loosely packed collagen is

significantly increased, providing a window for effective intervention. The ability to prevent or mitigate fibrosis through pharmacologic or regenerative medicine approaches may benefit from this through understanding of the variations in the ECM and muscle microenvironment following VML injury.

Cross-sections of the mid-belly of the TA muscle, directly at the mid-point of the VML injury, were histologically stained to evaluate collagen organization and alignment. While the total area of both densely and loosely packed collagen fractions were significantly increased 21 days post-VML injury throughout the muscle, the average ratio between the two (dense:loose) was declined relative to control, suggesting a more disorganized ECM during the remodeling phase after injury. This increased proportion of loosely packed collagen is seemingly in agreement with findings by Smith et al. in a model of muscular dystrophy, in which collagen organization changes throughout the pathology.[168] Yet in the defect and border regions loosely packed collagen was reduced after 48 days, and densely packed collagen significantly increased creating a more uniformed organization. Densely packed collagen possibly influences overall stiffness of the muscle tissue,[168] which has been shown to increase over time following VML.[132] Curiously, that trend was not observed in the remaining muscle, which maintained a heterogenous collagen organization through 48 days post-VML. However, an alternative interpretation of polarized hue differences is that the intensity of staining indicates collagen thickness, or degree of alignment, while hue indicates direction of collagen fiber alignment relative to the incident light.[169] Given a cross-section of muscle fibers should be in alignment with the incident light, hue difference can then give an approximation of collagen fiber alignment relative to muscle fiber alignment, with red/orange hues of collagen at a larger angle to the muscle fiber and green hues aligned more parallel to muscle fibers.[170] Taken with this interpretation, our findings indicate there is more collagen aligning parallel to the longitudinal axis of muscle fibers at the early time points post-VML, based on the increased ratios of green to red/orange/yellow. By aligning longitudinally, collagen would bear more of the stress applied to the muscle and take on a greater functional role in force transmission, which was observed in the remaining muscle

through 48 days. Conversely the increased proportion of red/orange/yellow in the defect and border regions would indicate collagen alignment at a larger angle relative to the longitudinal muscle axis, likely decreasing tensile strength of the muscle.

All biochemical measurements of protein contents were taken in the remaining (i.e., uninjured) muscle directly distal to the defect area from 3-48 days following VML injury. Therefore, data herein indicate significant ECM changes are not confined to the site of injury and immediate surrounding area, but which are pervasive throughout the muscle. Recent work by Southern et al. further supports this notion as substantial disruptions in the mitochondrial network throughout the remaining muscle were observed well into the uninjured muscle.[129] Moreover, as expected the proportion of collagen III in the remaining muscle increased during early time points post-VML. However, rather unexpected was the seemingly synchronized increase in collagen I and reduction in collagen III by 48 days post-VML. The switch in collagen proportions could highlight an important phase in wound healing for intervention. The late increase in collagen I likely indicates a more heavily cross-linked ECM in the remaining muscle, and taken together with histologic findings, a more mature densely packed fibrotic scar tissue in the defect area. Addressing pathologic fibrosis through pharmacologic administration would preferably occur before fibrosis becomes denser, as heavily packed and cross-linked collagen is harder to degrade.

Both histologically and biochemically CCN2/CTGF was quantified to assess its role in fibrotic signaling after VML injury. Importantly, CCN2/CTGF is thought to act synergistically, yet downstream of TGF-β1 in the development of fibrosis. TGF-β1 is a popular anti-fibrotic target due to its well-known role as a pro-fibrotic factor; however, it also plays an important role in the myogenic response and thus preserving TGF-β1 signaling may prove beneficial.[171,153] To that extent, this study found robust increases in CCN2/CTGF at all time points through 48 days post-VML, suggesting it could be an ideal target for inhibition of fibrosis independent of TGF-β1. Indeed, a monoclonal antibody that inhibits CCN2/CTGF, known as Pamrevlumab, was evaluated in *mdx* mice as an anti-fibrotic treatment.[150] It was

observed to reduce fibrosis, increase muscle strength, and improve satellite cell grafting ability. Anti-fibrotic drugs used in VML animal models have similarly been successful in the reduction of fibrosis, but unexpectedly led to a reduction of force output.[133,132] The loss in force from fibrotic reduction underlines the role of fibrosis in force transmission, or functional fibrosis as previously alluded.[136] Therefore, treatments successful in reducing fibrosis would likely need to be coupled with a subsequent myogenic treatment to maintain and eventually increase force output and muscle function.

Recently, we highlighted the ability of autologous minced muscle grafts to aid in muscle regeneration post-VML injury, yet muscle strength deficits were not able to fully recover.[137] Transcriptome profiling revealed similar overexpression of inflammatory and fibrotic genes in muscle graft treated and non-treated groups, suggesting myogenic treatments alone do not mitigate these processes but remain hindered by them. Furthermore, Boldrin et al. demonstrated that satellite cells derived from *mdx* mice retain their regenerative capabilities when placed in a permissive microenvironment.[172] This would suggest that, at least in part, the accumulation of ECM components in fibrotic muscle inhibits regenerative capabilities of endogenous satellite cells as well as injected stem cells or muscle grafts. Therefore, restoring the ECM to a permissive state would allow greater likelihood of successful satellite cell engraftment, in addition to improved endogenous regeneration. The observation of a significant reduction in decorin content from 14 to 48 days post-VML reveals a potential treatment avenue in that regard. Decorin is known to regulate collagen fibrillogenesis by binding to collagen I,[67] and also has binding sites for CCN2/CTGF, TGF-β1 and myostatin.[173] Thus, it has the ability to simultaneously inhibit fibrotic signaling while stimulating myogenic signaling.

In conclusion, in a rat model of VML injury the early wound healing phase was highlighted by increased proportions of collagen III and loosely organized collagen, presumably following a normal muscle repair process of transient ECM remodeling. However, these observations were abruptly transformed by 48 days post-VML into a muscle dominated by collagen I and a heavily aligned and densely

packed collagenous defect area. Future work will seek to mitigate this fibrotic transition by the inhibition of CCN2/CTGF. Additionally, evaluating ECM changes at more time points between 14 and 48 days may allow for optimal timing of pharmacological intervention. As the initial inflammatory and remodeling phase is quite important in successful wound healing, timing of anti-fibrotic administration and any subsequent myogenic treatment will likely be critical, with a slightly delayed treatment possibly offering superior outcomes.

Chapter 3: Conclusions and Future Directions

Comparing our findings to known models of skeletal muscle fibrosis reveals interesting similarities and differences. The early ECM alterations in the remaining muscle following VML injury in many ways resemble features that are present in dystrophic muscle. For example, both appear to increase the relative proportion of collagen III, increase proteoglycan content, and shift to a matrix that is more loosely packed. Given that much of the literature regarding skeletal muscle fibrosis uses a dystrophic muscle model, these similarities were expected. Intriguingly, the eventual shift to a collagen I dominated matrix coupled with a decrease in decorin content in the remaining muscle following VML was not anticipated. Yet wound healing in other types of tissue, such as skin, does follow a similar progression of collagen III to increasing collagen I over time, especially in scar formation.[174] Thus it almost seems as if dystrophic muscle is stuck in the early phase of wound healing, supported by the fact that it is continually being re-injured; whereas VML pathology advances past successful healing into a state of densely packed and heavily cross-linked matrix which equally limits function.

Future directions include evaluating various treatment options drawn upon the results of our current study. CCN2/CTGF is a target candidate for inhibition based on our findings of robust and persistent overexpression after VML injury. Moreover, the timing of such anti-fibrotic interventions may be critical, as the early inflammatory signaling and transient ECM deposition is needed for proper healing. Additionally, combining anti-fibrotic treatment with a subsequent myogenic treatment such as a muscle graft has the potential to augment results. Our current findings also highlight decorin as a promising potential therapeutic option. Previous studies using decorin injection after muscle laceration injuries found it reduced scar tissue formation and significantly increased strength relative to injured control muscle.[175,70] Moreover, recent work in our lab indicated significant progressive denervation in the remaining muscle following VML injury. Exploring the role between increased denervation and fibrosis may be interesting. As previously noted, biglycan content is altered in CP, which is possibly a factor in the disrupted NMJ's associated with the disease. Determining the direction of that relationship,

whether fibrosis drives denervation or vice versa, may be useful in optimizing treatment approaches.

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